University of Bath

PHD

Dermatopharmacokinetics and pharmacodynamics of topical glucocorticoids

Wiedersberg, Sandra

Award date:
2006

Awarding institution:
University of Bath

Link to publication

Alternative formats
If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (https://creativecommons.org/licenses/by-nc-nd/4.0/). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy
If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

Download date: 05. May. 2023

DERMATOPHARMACOKINETICS AND PHARMACODYNAMICS OF TOPICAL GLUCOCORTICOIDS

SANDRA WIEDERSBERG

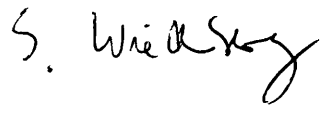
A thesis submitted for the degree of Doctor of Philosophy

University of Bath
Department of Pharmacy and Pharmacology
November 2006

COPYRIGHT

Attention is drawn to the fact that copyright of this thesis rest with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purpose of consultation.



UMI Number: U223116

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U223116
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF BATH
LIBRARY
12 JUL 2007
Ph.D.

Acknowledgments

I would like to express my gratitude to all the persons involved in the realization of this work, for their scientific, technical, financial and moral contribution.

- Professor **Richard H. Guy**, the final supervisor of my thesis, for taking over the responsibility for me and the project, for giving me the opportunity to work in his research group in Archamps and finally here in Bath, for encouraging me in the fascinating field of skin research, for his confidence, numerous stimulating discussions, and his motivating support throughout my studies.

- Professor **Claudia S. Leopold**, the supervisor during the first years of my studies, for giving me the opportunity to work in her research group at the University of Leipzig, and for her constructive scientific discussions.

- Doctor **Charareh Pourzand** and Professor **Jonathan Hadgraft**, for accepting to evaluate this work.

- All the **volunteers**, for participating in the experiments, their fidelity and patience.

- All my **friends and colleagues** from the University of Bath, for their personal and technical support, and for their contribution to a very enjoyable and friendly working atmosphere.
  Especially, I would like to thank **Jean-Philippe**, for his helpful input and for his continued interest throughout the last 2 years; and **Begoña,** for her support in statistics and in the delivery of 'caffeine'.

- My friends **Anke, Sara, Karin** and **Sandra,** for their useful scientific discussions and for their moral support throughout the last years.

- My **parents** and my **brother**, for their encouragement at all times.

Table of contents

DERMATOPHARMACOKINETICS AND PHARMACODYNAMICS OF TOPICAL GLUCOCORTICOIDS

Abstract

The efficiency of topical drug delivery is notoriously poor, with typical bioavailabilities of only a few percent of the applied dose. A major reason for this disappointing situation is the absence of a quantitative and validated methodology (apart from the vasoconstrictor assay for topical glucocorticoids) with which to quantify the rate and extent of drug delivery to a target into the skin. In an attempt to address this situation, significant efforts are being directed to the dermatopharmacokinetic (DPK) approach using tape-stripping.

The main objective of this thesis, therefore, was to compare the *in vivo* bioavailability profiles of betamethasone 17-valerate (BMV) assessed using the vasoconstrictor assay and the DPK approach. Furthermore, the ability of these two methods to distinguish between different formulations as well as different concentrations was examined. As the DPK approach is currently under critical re-evaluation, different cleaning procedures of the skin before tape-stripping were compared and evaluated. Moreover, the influence of the viscosity of the formulation on the DPK results was also determined. In addition, the effect of the vehicles on skin hydration was studied.

Applying different BMV concentrations resulted in a clear concentration dependence of the skin blanching response until saturation of the response occurred. Upon this saturation effect, any changes between different formulations and between concentrations could no longer be observed. Due to the saturable nature of the skin blanching response, the interpretation of the data has to be considered with care and, therefore, it is important to operate in the linear part of the 'dose-response' curve, whenever quantitative conclusions about bioavailability are to be drawn. The DPK approach, on the other hand, showed reasonable reproducibility and distinguished clearly between different formulations and different BMV concentrations applied; it appears to offer a reliable metric, therefore, with

which to quantify topical bioavailability. The cleaning procedure, as well as the viscosity of the formulation applied, have a significant influence on the apparent extent of drug delivery into the stratum corneum. Excess formulation, especially from semi-solids, may be trapped in the skin 'furrows' and requires an efficient skin cleaning procedure to ensure its complete removal.

Overall, the DPK technique merits continued evaluation and optimization as a tool for the quantification of topical bioavailability and bioequivalence. These steps are essential for the ultimate objective of validating the results of DPK experiments against credible clinical data.

List of abbreviations

Δa_{max}	Maximum blanching response
A	Amount of drug
AARC	Area above the response curve
A_{max}	Maximum amount of drug
ANOVA	Analysis of variance
AUC	Area under the curve
BA	Bioavailability
BE	Bioequivalence
BMV	Betamethasone 17-valerate
$C_{s,SC}$	Saturation level in the SC
$C_{s,V}$	Saturation level in the vehicle
C_V	Concentration in the vehicle
C_x	Concentration within the barrier
D/L^2	Diffusivity parameter across the barrier
DPK	Dermatopharmacokinetic
EF	Enhancement factor
Eq.	Equation
FDA	Food and Drug Administration
HPLC	High-performance liquid chromatography
K	Partition coefficient
k_0	Input rate constant
k_e	Elimination rate constant
L	Pathlength of the barrier
LMO	Light mineral oil
MCT	Medium chain triglycerides
ME	Microemulsion
SC	Stratum corneum
SD	Standard deviation
T	Time
TCL	Transcutol®
TEWL	Transepidermal water loss

TG	Topical glucocorticoids
T_{max}	Time point at which a maximum was attained
v/v	Ratio of volumes
VA	Vasoconstrictor assay
w/w	Ratio of weights
x	Position within the barrier

CHAPTER 1

Bioavailability and bioequivalence of topical glucocorticoids

Bioavailability and bioequivalence of topical glucocorticoids

Sandra Wiedersberg[1], Claudia S. Leopold[2], Richard H. Guy[1]

[1]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK.

[2]Institute for Pharmacy, Department of Pharmaceutical Technology, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany.

Review paper to be submitted

1. Introduction

Topical glucocorticoids (TG) are the most frequently prescribed drugs by dermatologists. Their clinical effectiveness in the treatment of psoriasis and atopic dermatitis is related to their vasoconstrictive, anti-inflammatory, immunosuppressive and antiproliferative effects. Treatment with TG formulations is effective, easy to administer, acceptable to patients and safe when used correctly. This review focuses on the main factors influencing the effectiveness and bioavailability of TG namely (i) the structure of the skin barrier, (ii) effects and side-effects of TG, (iii) chemical modifications in the TG structure, (iv) vehicle or formulation effects, and (v) methods to determine bioavailability and/or bioequivalence (BA/BE).

2. The skin barrier

The skin is the largest organ of the body with an area of approximately 2 m^2 and is the interface between the organism and its environment. It prevents the loss of water and the ingress of foreign materials. In essence, the skin consists of three functional layers: the epidermis, the dermis (corium) and the hypodermis. The hypodermis is a subcutaneous tissue consisting of fat and muscle and acts as a heat isolator, a shock absorber, and an energy storage region. The dermis is ~ 2 mm thick and contains collagen, elastic fibres, blood vessels, nerves as well as hair follicles and sebaceous and sweat glands. The main cells in the dermis are fibroblasts, which are involved in the immune and inflammatory response and upon which glucocorticoid receptors are found. The dermis is the source of nutrients for the epidermis. Because the epidermis is avascular, essential substances are transported only by passive diffusion. The epidermis has a multilayered structure reflecting different stages of differentiation of the skin cells (the keratinocytes). From the proliferative, basal layer, the cells change in an ordered fashion from metabolically active and dividing to dense, functionally dead, and fully keratinised, the so-called corneocytes. These corneocytes are embedded in a lipid matrix and form the outer 10 - 20 µm of the epidermis, the stratum corneum (SC) [1].

The cytoplasm of cultured human skin keratinocytes and fibroblasts contains macromolecules that bind glucocorticoids with high affinity, suggesting that the sites of action for TG are both epidermal and dermal cells [2,3]. To reach these target cells, TG have to permeate the SC (Figure 1), which contributes the major barrier to percutaneous absorption [4,5].

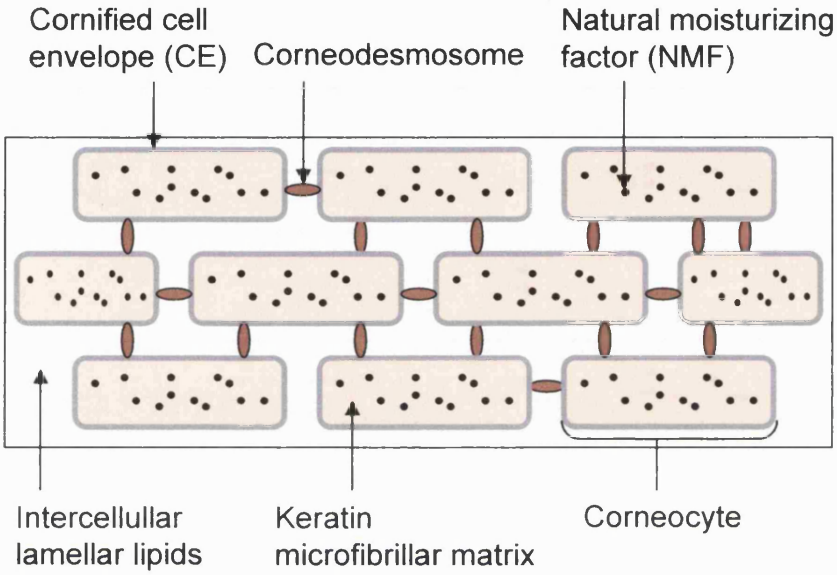


Figure 1: Schematic 'bricks and mortar' representation of the structural and functional components of the SC. Modified from Harding [6].

The terminally differentiated corneocytes (bricks) consist primarily of a highly organized keratin microfibrillar matrix, which provides mechanical resistance. Natural moisturizing factor (NMF), a mixture of amino acids, lactic acid, urea, citrate and sugar, is present at a high level in the corneocytes and acts as a very efficient humectant [7]. In addition, protein junctions, the corneodesmosomes, link adjacent corneocytes and ensure the cohesiveness of this layer [26-28]. During the formation and maturation of the SC, desmosomes are modified and their number decreases towards the skin surface [27,28]. Each corneocyte is surrounded by a 15 - 20 nm thick protein shell – the cornified cell envelope (CE), a 15 nm layer of defined structural proteins and a 5 nm thick layer of specialized lipids. The lipid monolayer provides a hydrophobic interface between the CE itself and SC lipid lamellae and helps maintain water barrier function [8,9]. While typical biological

membranes are mainly composed of phospholipids, the intercellular SC lipids (mortar) comprise primarily ceramides (~ 40 % w/w), free fatty acids (~ 10 % w/w) and cholesterol (~ 25 % w/w), together with a small fraction of cholesterol sulfate and triglycerides (Figure 2) [10-13]. The lipids originate from lamellar bodies that are synthesized in the upper viable layers of the epidermis, which are ultimately secreted from the cells into the intercellular space. These lipids, which are organized in multilamellar bilayers, regulate the passive flux of water through the SC and are considered to be very important for skin barrier function [14-19].

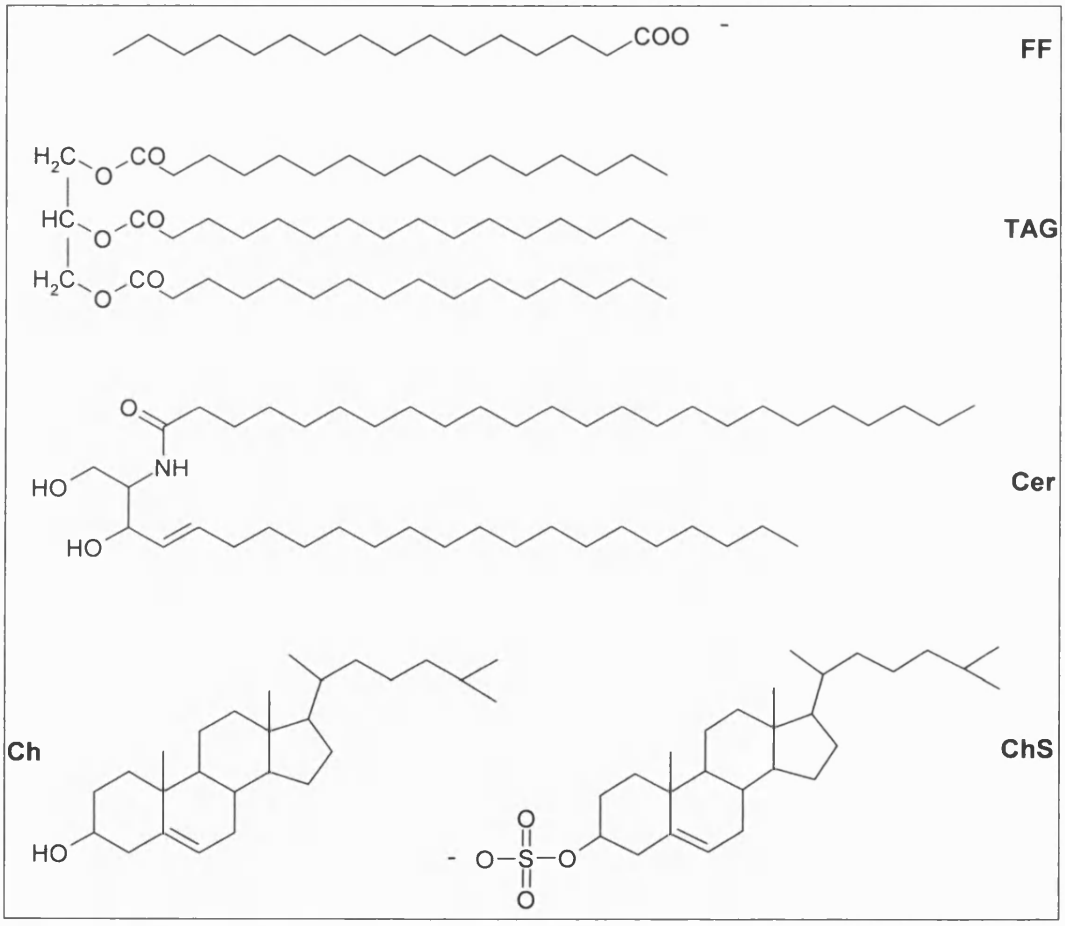


Figure 2: Basic structures of SC lipids: free fatty acids (FF), triglycerides (TAG), ceramides (Cer), cholesterol (Ch), cholesterol sulfate (ChS).

Nine classes of ceramides have been recognized and differ from each other by the head group architecture and by the average fatty acid chain length [20,21]. Ceramides 1 and 9 are believed to be particularly important

[22-24]. A direct relationship between the degree of barrier perturbation (measured as transepidermal water loss) and the amount of SC lipid removed has been demonstrated [25].

This 'brick and mortar' arrangement of the SC creates a tortuous route for compounds to permeate the barrier.

3. Percutaneous absorption and mechanism of TG action

The percutaneous absorption of a drug from a topically applied formulation is a complex process [26]. The physicochemical characteristics of the drug and the vehicle and the physiological conditions of the skin can significantly affect percutaneous absorption. For a topically applied formulation containing a glucocorticoid the percutaneous absorption involves the following steps:

(a) release from the formulation,

(b) penetration into the skin's outermost layer, the SC and permeation/diffusion through the SC,

(c) partitioning from the SC into the viable epidermis and the dermis,

(d) within the viable epidermis/dermis, diffusion to reach the glucocorticoid receptor.

Penetration into the SC is assumed to be the rate-limiting step for percutaneous absorption of TG. In this case, the glucocorticoid amount reaching the target cell will be determined by its partitioning into and rate of transport through the SC. These processes are greatly influenced by the physicochemical properties of both the drug and the vehicle [27,28]. The *in vivo* clinical effectiveness of a TG formulation depends on the bioavailability of the glucocorticoid within the skin at the site of action. For TG the target cells are the keratinocytes and fibroblasts within the viable epidermis and dermis, where the glucocorticoid receptors are located [2,29]. Having attained the target, the cellular uptake and residence time of the steroid as well as its affinity for the glucocorticoid receptor will determine the clinical effect [3,30-32]. From studies with cultured human fibroblasts and keratinocytes, the cellular uptake of glucocorticoids has been shown to be a

non-mediated, passive diffusion process that involves two intrinsic steps: a rapid, non-specific, high-capacity association to the cell membrane followed by a slower process leading to strong binding of glucocorticoid within the cell [31]. The total uptake of steroid by fibroblasts and keratinocytes was related to drug lipophilicity. Although, as stated, steroids are generally thought to be transported across the cell membrane by passive diffusion, there is some evidence that certain target cells possess a specific transport system for these compounds [33]. The anti-inflammatory and immunosuppressive effects of TG seem to be mediated largely by regulation of corticosteroid-responsive genes. Within the cytoplasm, the steroid binds to the glucocorticoid receptor, forming a complex that is rapidly transported to the nucleus. The glucocorticoid-receptor complex inside the nucleus then binds to a region of DNA called the glucocorticoid responsive element to either stimulate or inhibit transcription and regulate thereby the inflammatory process [34,35].

In addition to this direct regulatory effect on gene transcription, TG are also able to indirectly regulate transcription by blocking the effects of other transcription factors, such as nuclear factor-kappa B alpha [36,37].

TG may inhibit the transcription of proinflammatory cytokine genes (including the interleukins IL-1, IL-2, IL-6, interferon gamma, and tumour necrosis factor-alpha genes), T-cell proliferation, and T-cell dependent immunity [38]. In fibroblasts, IL-1α is responsible for proliferation, collagenase induction, and IL-6 synthesis, which control skin thickness [39]. The inhibition of IL-1α in keratinocytes has anti-inflammatory effects, whereas the same inhibition in fibroblasts has antiproliferative and atrophogenic effects. The vasoconstrictive effect of TG may contribute to their anti-inflammatory activity, diminishing erythema at the lesion site. However, the exact mechanism is not completely clear.

Finally, clinical efficacy is self-evidently and significantly influenced by corticosteroid structure, the formulation and the applied concentration of the drug [40-44]. These factors are described later in much greater detail.

4. Side-effects

Despite their clear benefit in the therapy of inflammatory disease, TG are associated with a number of side-effects that limit their use. One particularly important local side-effect is epidermal thinning or atrophy [45]. This effect can start after 3 to 14 days of corticosteroid treatment with microscopic degenerative changes in the epidermis, including reduction in cell size and number of cell layers [34,46]. TG inhibit epidermal cell differentiation by inhibition of keratinocyte proliferation and acceleration of keratinocyte maturation [47-49].

Moreover, prolonged TG therapy increases basal transepidermal water loss, indicating an effect on permeability barrier function [46]. This change has been associated with a decrease in SC thickness, a reduction in lipid content [46], a decrease in the number of lamellar bodies [48] and in the number of intercellular lamellae (although the structure of these lipid bilayers appeared normal [46]).

Even short-term treatment with a potent TG, clobetasol (0.05 % w/V), applied once a day for 3 days can alter epidermal structure and function in humans [50]. SC barrier recovery was significantly delayed, even though visible changes in the epidermis were not observed [50]. In hairless mouse skin, the same short term treatment inhibited epidermal cholesterol, fatty acid, and ceramide synthesis by more than 50 %. This inhibition may account for the decreased production and secretion of lamellar bodies and the impaired formation of lipid bilayers in the SC and the resulting abnormality in barrier function [50]. TG also exerted negative effects on the integrity and cohesion of the SC owing to a reduction in the number of corneodesmosomes in the SC [50].

Furthermore, histological changes are observed in the dermis. Dermal atrophy results from the direct antiproliferative action of TG on fibroblasts [51]; in turn, this leads to a reduction in the synthesis of collagen and mucopolysaccharides and a loss of dermal support. The elastin fibres in the upper layers of the dermis become thin and fragmented, while the deeper fibres collapse to form a compact and dense network. As a result of this thin

and brittle skin, there is local vascular dilatation, which is responsible for striae, telangiectasia, and purpura [34,52].

Systemic side-effects of TG, such as pituitary-adrenal axis suppression, are rare but have to be seriously considered when treating children because of the potential for growth retardation. Furthermore, children have a higher ratio of total body surface area to body weight (about 2.5- to 3-fold that of adults). The degree of adrenal suppression increases with the potency and concentration of the TG, application area, occlusion and degree of inflamed skin. Other systemic side-effects include Cushing's syndrome, the aggravation of diabetes mellitus, and increasing or causing hypertension and osteonecrosis.

5. Glucocorticoid chemistry

In humans, the naturally occurring corticosteroid is cortisol, or hydrocortisone, which is produced primarily in the adrenal gland. The majority of TG that are used therapeutically are synthetic derivatives of hydrocortisone (Figure 3). Hydrocortisone has an androstane structure arranged in four rings, with a relative low potency and a short duration of action. The free hydroxyl-group at C-11 is essential for the pharmacological effect. Increased glucocorticoid activity (increased affinity for the corticosteroid receptor) can be achieved by introduction of an additional double bond at C-1 and substitution at the C-16 position. The lipophilicity of the steroid and the duration of action are greatly increased by fluorination of the B ring at the C-9 and/or C-6 position. Moreover the lipophilicity and metabolic resistance of TG may also be increased by adding ester or acetal groups to the D-ring (e.g. betamethasone 17-valerate). Clobetasol propionate is the most potent of the currently available TG.

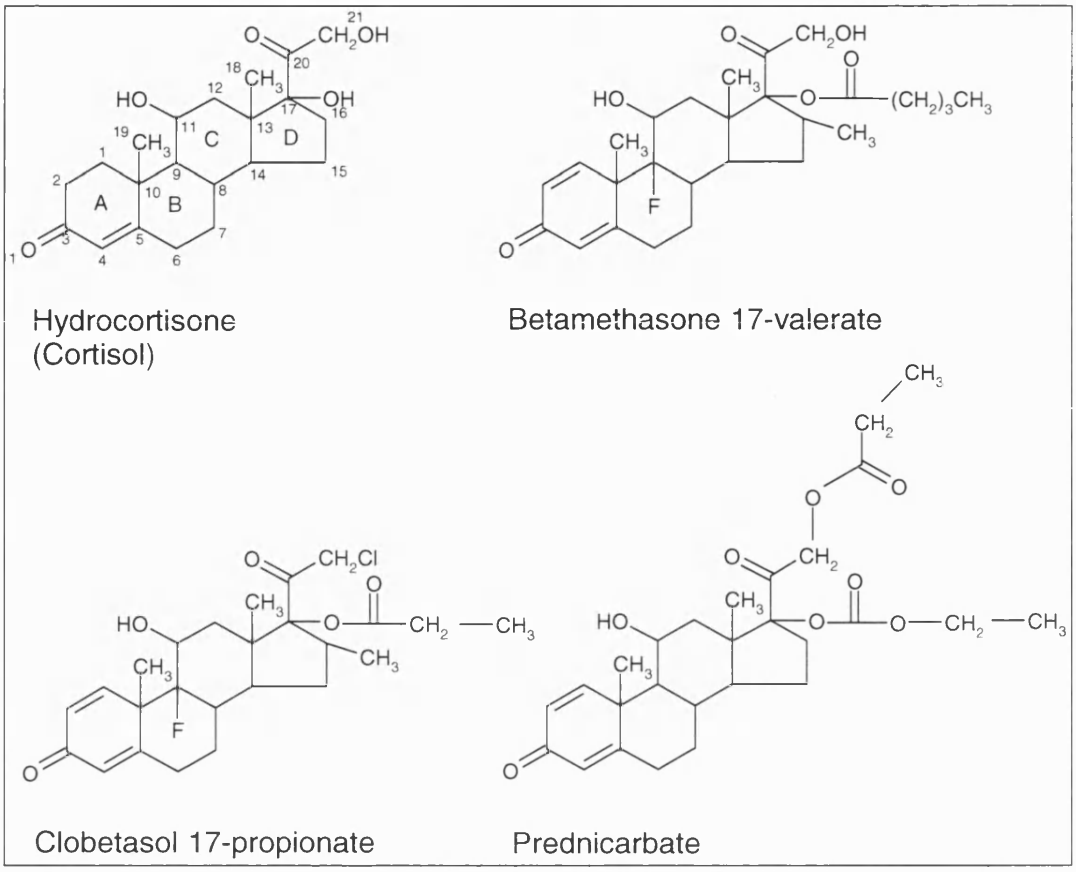


Figure 3: Structure of hydrocortisone and selected TG of greater potency.

Strategies to optimise the potency and, in particular, the anti-inflammatory and immunosuppressive capacity of TG, while minimizing their adverse effects have been pursued. But 'ideal' TG have not yet been synthesized. Prednicarbate, hydrocortisone aceponate, mometasone furoate and methylprednisolone aceponate are the first, so-called soft-steroids. These are 17,21-double esters of hydrocortisone with significant anti-inflammatory activity, but with the least capacity to induce skin atrophy [53-56]. The relative weak side-effects are the result of a specific metabolic step and a selective influence on cytokine production.

In vivo, mometasone furoate, methylprednisolone aceponate and hydrocortisone induced similar skin atrophy but the soft steroids elicited a much greater blanching response [57]. Similarly, prednicarbate caused significantly less skin atrophy than the equipotent betamethasone 17-valerate [58]. This low atrophogenic effect is the result of its minor effects on IL-1α and IL-6 suppression in dermal fibroblasts; the high degree of IL-1α

suppression in epidermal keratinocytes, on the other hand, is almost equivalent to that of betamethasone 17-valerate [39,59,60]. Prednicarbate (PC) penetrates the epidermis more readily due to esterification at position 17 and 21. Reaching the keratinocytes, PC is rapidly metabolised to prednisolone 17-ethylcarbonate (PEC), which is believed to be primarily responsible for anti-inflammatory effect due to its higher receptor affinity. In fibroblasts, PEC inhibits IL-1α and IL-6 much more than PC. However, the metabolism of PC in fibroblasts is only 1 % per hour, possibly due to low esterase activity. Moreover, the permeation of PC through the epidermis into the dermis is very slow, resulting in a negligible atrophogenic effect [60,61]. Therefore, these compounds can be used to treat sensitive areas, such as the face, and large surface areas in children, with minimal local and systemic side-effects [54]. Fluticasone propionate (Figure 4), a fluoromethyl androstane-17β-carbiothioate, is another new soft steroid with good anti-inflammatory activity but a much lower potential to cause systemic side-effects. The two esterifications at positions 17 and 20 increase the molecule's lipophilicity, and its uptake by and affinity for the glucocorticoid receptor. The small amount of drug that is systemically absorbed is rapidly metabolised to the inactive carboxylic acid derivative in the liver [62].

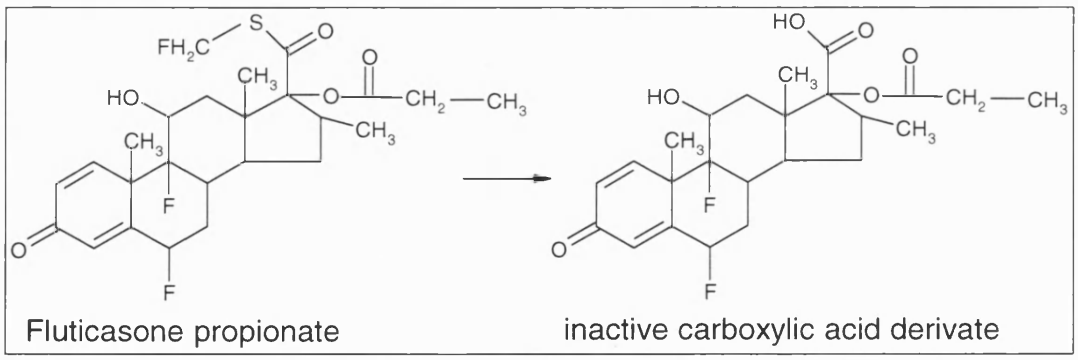


Figure 4: Fluticasone propionate and its inactive metabolite.

6. Potency/Classification

The efficacy of a TG is related to its pharmacological potency and to its ability to be absorbed into the target cells within the viable epidermis and dermis [63]. Potency is a complex function of the physical and chemical properties of both the drug and its vehicle [42,64]. For the TG, a ranking of drugs and vehicles has been evolved using the skin blanching assay.

Table 1: Classification of TG according to potency by the National Psoriasis Foundation (from www.psoriasis.org).

TG PRODUCT	INCORPORATED TG
CLASS I – Superpotent	
Clobex Lotion, 0.05%	Clobetasol propionate
Cormax Cream/Solution, 0.05%	Clobetasol propionate
Diprolene Gel/Ointment, 0.05%	Betamethasone dipropionate
Olux Foam, 0.05%	Clobetasol propionate
Psorcon Ointment, 0.05%	Diflorasone diacetate
Temovate Cream/Ointment/Solution, 0.05%	Clobetasol propionate
Ultravate Cream/Ointment, 0.05%	Halobetasol propionate
CLASS II – Potent	
Cyclocort Ointment, 0.1%	Amcinonide
Diprolene Cream AF, 0.05%	Betamethasone dipropionate
Diprosone Ointment, 0.05%	Betamethasone dipropionate
Elocon Ointment, 0.1%	Mometasone furoate
Florone Ointment, 0.05%	Diflorasone diacetate
Halog Ointment/Cream, 0.1%	Halcinonide
Lidex Cream/Gel/Ointment, 0.05%	Fluocinonide
Maxiflor Ointment, 0.05%	Diflorasone diacetate
Maxivate Ointment, 0.05%	Betamethasone dipropionate
Psorcon Cream 0.05%	Diflorasone diacetate
Topicort Cream/Ointment, 0.25%	Desoximetasone
Topicort Gel, 0.05%	Desoximetasone
CLASS III – Upper Mid-Strength	
Aristocort A Ointment, 0.1%	Triamcinolone acetonide
Cutivate Ointment, 0.005%	Fluticasone propionate
Cyclocort Cream/Lotion, 0.1%	Amcinonide
Diprosone Cream, 0.05%	Betamethasone dipropionate
Florone Cream, 0.05%	Diflorasone diacetate

Lidex-E Cream, 0.05%	Fluocinonide
Luxiq Foam, 0.12%	Betamethasone valevate
Maxiflor Cream, 0.05%	Diflorasone diacetate
Maxivate Cream/Lotion, 0.05%	Betamethasone dipropionate
Topicort Cream, 0.05%	Desoximetasone
Valisone Ointment, 0.1%	Betamethasone valerate
CLASS IV - Mid-Strength	
Aristocort Cream, 0.1%	Triamcinolone acetonide
Cordran Ointment, 0.05%	Flurandrenolide
Derma-Smoothe/FS Oil, 0.01%	Fluocinolone acetonide
Elocon Cream, 0.1%	Mometasone furoate
Kenalog Cream/Ointment/Spray, 0.1%	Triamcinolone acetonide
Synalar Ointment, 0.025%	Fluocinolone acetonide
Uticort Gel, 0.025%	Betamethasone benzoate
Westcort Ointment, 0.2%	Hydrocortisone valerate
CLASS V - Lower Mid-Strength	
Cordran Cream/Lotion/Tape, 0.05%	Flurandrenolide
Cutivate Cream, 0.05%	Fluticasone propionate
DermAtop Cream, 0.1%	Prednicarbate
DesOwen Ointment, 0.05%	Desonide
Diprosone Lotion, 0.05%	Betamethasone dipropionate
Kenalog Lotion, 0.1%	Triamcinolone acetonide
Locoid Cream, 0.1%	Hydrocortisone butyrate
Pandel Cream 0.1%	Hydrocortisone probutate
Synalar Cream, 0.025%	Fluocinolone acetonide
Uticort Cream/Lotion, 0.025%	Betamethasone benzoate
Valisone Cream/Ointment, 0.1%	Betamethasone valerate
Westcort Cream, 0.2%	Hydrocortisone valevate
CLASS VI – Mild	
Aclovate Cream/Ointment, 0.05%	Alclometasone dipropionate
DesOwen Cream, 0.05%	Desonide
Synalar Cream/Solution, 0.01%	Fluocinolone acetonide
Tridesilon Cream, 0.05%	Desonide
Valisone Lotion, 0.1%	Betamethasone valerate
CLASS VII - Least Potent	
Topicals with hydrocortisone, dexamethasone, methylprednisolone and prednisolone	

The American classification includes seven potency groups (Table 1) [65], while the British National Formulary recommends only four (Table 2) [66]. In the former system, the potency of a product is characterized by the corticosteroid, its concentration and the nature of the vehicle. Corticosteroid formulations in the same potency group have similar efficacy and a similar potential to provoke side-effects. That is, the greater the potency, the greater the therapeutic efficacy, but also the greater the adverse effects. Low-potency formulations are considered acceptable for long-term treatments while the more potent products should be reserved for shorter regimes and for use at sites, such as the palms and soles, where low potency corticosteroids are ineffective [67]. The British classification system is made irrespective of the topical vehicle used.

Table 2: Classification of TG by potency according to the British National Formulary (BNF).

POTENCY	% w/w	TG
Mild		Hydrocortisone
	1	Hydrocortisone acetate
	0.25	Methylprednisolone
	0.05	Aclometasone dipropionate
	0.01-0.1	Dexamethasone
	0.0025	Floucinolone acetonide
	0.75	Fluocortyn butyl ester
	0.5	Prednisolone
Moderate	0.05	Clobetasone butyrate
	0.02	Triamcinolone acetonide
	0.005	Fluocinolone acetonide
Potent	0.05	Betamethasone diproprionate
	0.1	Betamethasone valevate
	0.025	Fluocinolone acetonide
	0.1	Hydrocortisone 17-butyrate
	0.05	Halometaone monohydrate
	0.1	Diflucortolone valevate
Very Potent	0.1	Halcinonide
	0.05	Clobetasol propionate

The dependence of the pharmacological response on the drug concentration in the vehicle is classically illustrated by a 'dose-response' curve (Figure 5) [68]. The profile is characterized by (i) a threshold concentration S, which is the minimum necessary to induce a response, (ii) a range over which the response increases linearly with the logarithm of the 'dose', (iii) the EC_{50}, the concentration that elicits 50 % of the maximal effect, and (iv) a plateau (E_{max}), where further increases in concentration provoke no additional pharmacological response. Clearly, different formulations of the same steroid can shift the position of the 'dose-response' curve by either enhancing or retarding the drug's penetration into the skin. The bioavailability factor f can be estimated from the horizontal distance between parallel curves.

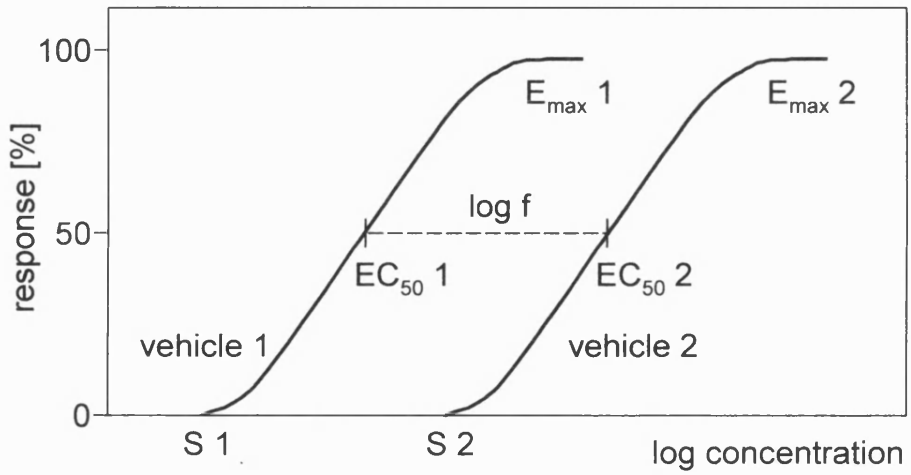


Figure 5: Typical 'dose-response' curves of two vehicles.

In the early 1970s, Katz and Poulsen described the significance of both release (diffusion out of the vehicle) and penetration (diffusion into the skin barrier) for topical product design based on corticosteroids [69]. These two processes are dependent upon the physicochemical properties of both the drug and the vehicle [70].

The skin blanching response of betamethasone 17-benzoate increased proportionally with the concentration of the dissolved and diffusible drug, reaching the maximal response when the concentration equals the solubility of the drug in the vehicle (Figures 6 and 7) [71,72]. The blanching

response from suspensions of the drug was independent of the betamethasone 17-benzoate concentration [72]. In Figure 6 and 7, the blanching response of the test formulations (B_T) was related to the maximum blanching response of an internal standard (B_{ST}).

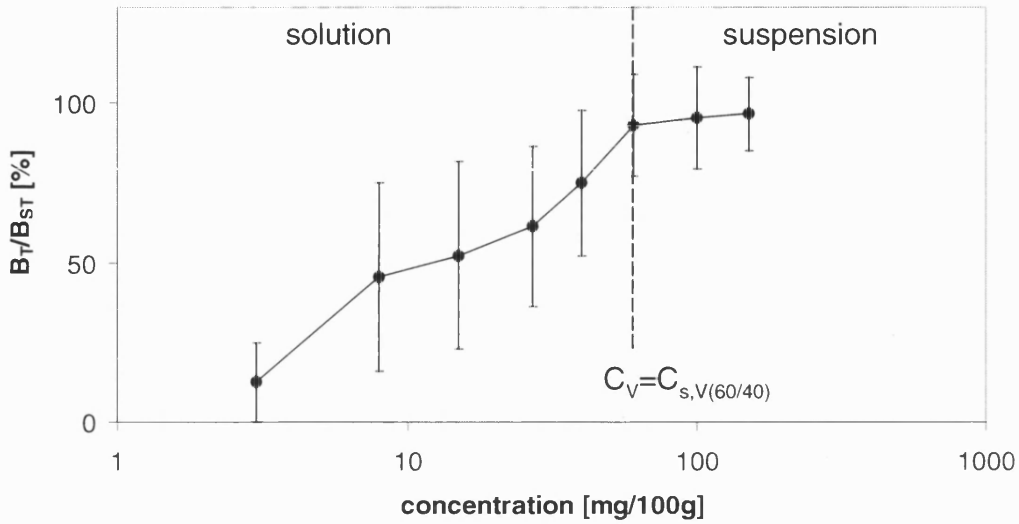


Figure 6: Dose response relationship of betamethasone 17-benzoate in neutral oil/paraffin 60/40. Means ± SD (n = 17-18). Applied concentrations: 3, 8, 15, 27, 40, 60, 100 and 150 mg/100g. (adapted from [72]).

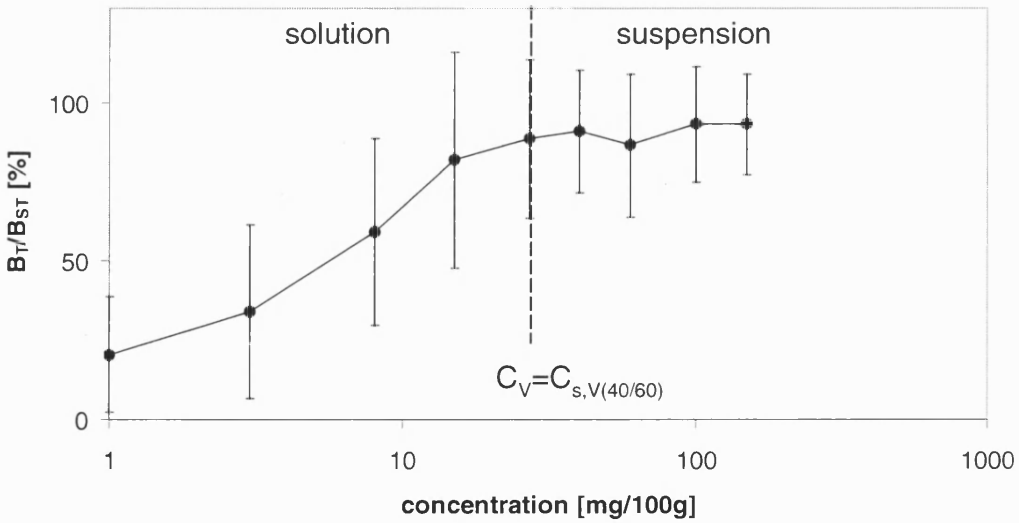


Figure 7: Dose response relationship of betamethasone 17-benzoate in neutral oil/paraffin 40/60. Means ± SD (n = 17-18). Applied concentrations: 1, 3, 8, 15, 27, 40, 60, 100 and 150 mg/100g (adapted from [72]).

The amount of drug needed for the same blanching response differs according to the steroid solubility in the vehicle. However, the same thermodynamic activity of the drug in the vehicle leads to the same pharmacodynamic response, provided that the formulation does not change either the solubility of the drug in the SC, nor its diffusivity across the SC.

7. Vehicles and formulations

TG are formulated in a variety of vehicles, including ointments, creams, lotions, gels and, more recently, foams. As mentioned above, the vehicle has a great influence on penetration into the SC and consequently on the bioavailability and potency of the glucocorticoid [73,74]. Ointment formulations are generally more potent than creams containing the same drug presumably due to their occlusive effect on the skin which may increase SC hydration and enhance drug transport [40,75,76]. Ointments are preferred for infiltrated, lichenified lesions, whereas creams are preferred for acute and subacute dermatoses. Lotions and gels are suitable for the treatment of scalp psoriasis. The novel, thermolabile, low-residue foam formulations, available for betamethasone 17-valerate and clobetasol propionate, are safe and effective in the treatment of psoriasis affecting scalp and nonscalp regions of the body. The foam formulations are associated with better patient compliance and improvements in quality of life [77,78].

The activity of a TG formulation can be enhanced by adding a chemical penetration enhancer, which may result in an increase of drug delivery into the SC.

Various studies, using a vasoconstrictor assay, have shown that huge differences existed between generic and original formulations containing the same glucocorticoid in the same concentration in different vehicles [42,79]. By altering the vehicle, betamethasone dipropionate, at a concentration of 0.05 %, has been formulated into four different potency groups (Table 1). Class I contains a cream (Diprolene® cream 0.05 %), which is a modified vehicle high in propylene glycol. Class II includes an ointment (Diprosone® ointment 0.05 %). Class III contains another cream (Diprosone® cream 0.05 %) with less propylene glycol then the class I formulation. Class V

incorporates betamethasone dipropionate in a lotion (Diprosone® 0.05 % lotion).

Kinetic considerations of the penetration process differentiate between solution and suspension-type formulations [80,81]. With suspensions the rate of penetration should be independent of the vehicle, on the solubility of the drug therein, and on the amount of incorporated drug as long as significant depletion does not occur over time [80]. However, in the case of solution-type formulations, the vehicle has an enormous influence on the rate of penetration. High solubility in the vehicle and a low partition coefficient between the SC and the vehicle lead to poor penetration of the drug into the SC and low bioavailability [69]. Therefore, it is important to know and control, where possible, the thermodynamic activity of the drug in the vehicle.

The dilution of commercially available TG formulations is a common practice, which causes problems. The expectation of the prescribing physician is that dilution reduces the efficacy of the corticosteroid formulation and can be adjusted to the needs of the patient. However, the extent to which efficacy is reduced is not always proportional to the degree of dilution. For example, a fluocinolone acetonide cream (Synalar® cream), when diluted by up to 10-fold, resulted in no significant reduction in potency as assessed by the vasoconstrictor assay [82]. A betamethasone 17-valerate ointment (Betnovate® ointment) was diluted by a factor of 32 with no statistically significant difference in the blanching response [83]. These results strongly suggest that the drugs in the original formulations were present as suspensions and that, even upon substantial dilution, there remained some undissolved drug present. In other words the thermodynamic activity stayed at its maximum level; it follows that drug delivery into the skin was not changed and the pharmacological response was unaltered. Had the drug been present in the original vehicle as a solution, dilution would have lowered the thermodynamic activity and led to lower permeation and a lesser response. Care must also be taken when diluting formulations with a base, which is not the same as the original vehicle. For example, Refai et al. showed that the *in vitro* permeation of hydrocortisone acetate is about 5 times lower after 1:2 dilution of Soventol® cream with a non-identical base

(Figure 8) [84]. Soventol® cream contains isopropyl alcohol and the penetration enhancer, isopropyl myristate. The reduced delivery is smaller than expected probably due to the reduced concentration of the enhancer in addition to the smaller thermodynamic activity of the drug.

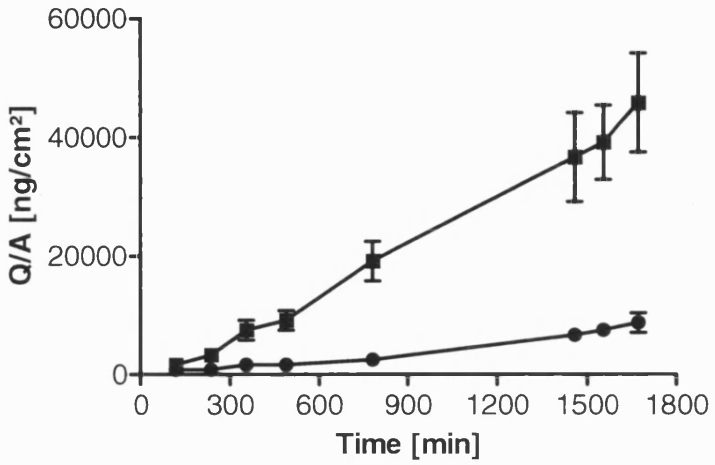


Figure 8: *In vitro* SC permeation of hydrocortisone from 1 % Soventol® cream (■), and Soventol® cream diluted with water-containing hydrophilic ointment 1:2 (●). Amount of drug permeating per unit area (Q/A) was plotted versus time (adapted from [84]).

Finally, it should be emphasized that the foregoing discussion is applicable specifically to intact, healthy skin, where penetration into and through the SC is the rate-limiting step. In the case of damaged skin, the release of drug from the formulation will determine uptake, and will be controlled by the characteristics of the vehicle.

8. Bioavailability/Bioequivalence testing

Bioavailability (BA) is defined as the "rate and extent to which the drug is absorbed from the formulation and becomes available at the site of action" (as stated in 21 CFR 320.1 [85]). Bioequivalence (BE) is defined as "the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives become available at the site of drug action when administered at

the same molar dose under similar conditions in an appropriately designed study" (as defined in 21 CFR 320.24 [85]). As mentioned above, for TG the sites of action are the glucocorticoid receptors in the viable epidermis and dermis. Typical bioavailabilities are only a few percent of the applied dose. Several *in vivo* and *in vitro* methods have been employed to assess the BA/BE of TG, and are summarised in Figure 9.

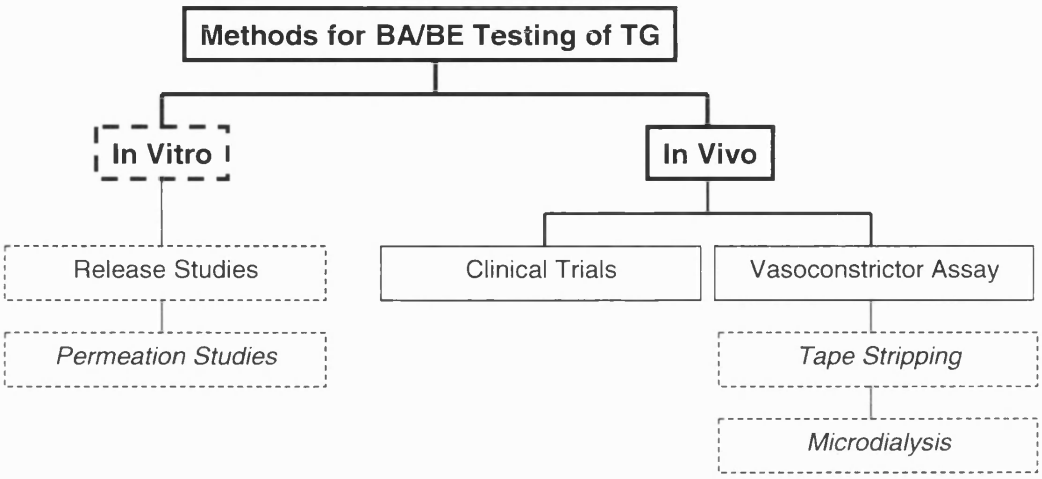


Figure 9: Methods for BA/BE testing of TG, italicised legends signify those methods, which are still under evaluation

For the moment, the only acceptable methods to assess BA/BE of topically applied drug formulations are clinical trials between generic and original products and pharmacodynamic response studies. Comparative clinical trials are considered to be the 'gold standard', but these studies are relatively insensitive, costly, time-consuming and require large numbers of subjects [86]. In contrast, pharmacodynamic response studies are relatively easy to perform, expose the subjects to only a small amount of the formulation for a short period of time, are fairly reproducible, and require a relatively small number of subjects [87]. The TG pharmacodynamic response is the ability to produce vasoconstriction of the microvasculature of the skin, leading to skin blanching (whitening) at the site of application. This "vasoconstrictor assay" was first described by McKenzie and Stoughton in 1962 [88,89]. Since that time, the method has been modified and extended to provide a reliable means to test TG and their formulations. The intensity of

skin blanching has been correlated with drug potency and the degree of drug delivery through the SC [90]. The blanching intensity has also been shown to correlate directly to clinical efficacy in patients with plaque psoriasis [40,75,91]. The vasoconstrictor assay has been used to measure the BA/BE of corticosteroid formulations in healthy volunteers [42,92,93] and has been adopted in 1995 for BE determination by the U.S. Food and Drug Administration (FDA), in a Guidance document "Topical Dermatologic Corticosteroids: *In Vivo* Bioequivalence" [87]. This Guidance counsels both pilot and pivotal studies. The preliminary, pilot study is performed to establish the dose duration-pharmacological response relationship of a reference listed drug (RLD). The formulation is applied for various times (dose durations) up to 6 hours to manipulate the amount of steroid delivered. At the end of the treatment period, the skin blanching response is measured with a chromameter over the next 24 - 28 hours. From the resulting response versus time profiles, the areas above the response curves (AARC) are calculated and plotted as a function of dose duration to obtain dose/response-like relationships (in accordance to Figure 5). From these profiles, the maximum AARC (= E_{max}) and the appropriate dose durations ED_{50}, D_1 and D_2 for use in the pivotal bioequivalence study are determined. ED_{50} is the dose duration required to achieve 50 % of E_{max}, D_1 and D_2 correspond to one-half ED_{50} and two times ED_{50}, respectively. The pivotal bioequivalence study then compares the *in vivo* response of the test product with that of the RLD using appropriate statistical tools to document whether bioequivalence has, or has not, been achieved.

There remains a concern, however, that the design and analysis of the pilot study can influence the findings from the pivotal test [94,95], the role of the pilot investigation is therefore crucial. A critical factor, which is not specified in the Guidance, is the volume of formulation to be applied *in vivo*. It has been reasonably argued that the applied vehicle volume should be the same at all sites to ensure accurate and meaningful bioavailability data [95]. The situation becomes complex for preparations in which the drug is in solution and may therefore deplete over time, and their comparison with suspension-type formulations [95,96].

The chromameter has been adopted by regulatory agencies, such as the U.S. FDA, as the current standard for the measurement of corticosteroid-induced skin blanching. The chromameter quantifies the reflectance of a xenon source light pulse in terms of three measures: the L-scale (light-dark), the a-scale (red-green) and the b-scale (yellow-blue). These three values can be used to define a point in three-dimensional space that characterizes a colour in absolute terms. The Guidance protocol suggests the use of only the a-scale values in quantifying the blanching response. The chromameter is viewed as an 'objective' measurement device compared to 'subjective' visual scoring. There are several reports comparing the chromameter with the visual technique [90,97-99], with other reflectance instruments [100], with laser Doppler velocimetry or, more recently, with digital image analysis [101,102]. Although, the chromameter has been adopted by the FDA as the current standard method for topical BE testing, it has been criticized for different reasons [94,97,103] and there are continuing efforts to examine alternative approaches. Nevertheless, despite its limitations, the vasoconstrictor assay remains the standard procedure to assess the BA/BE of TG.

Other pharmacodynamic effects that may be quantified are the vasodilatation (erythema) and skin temperature increase induced by nicotinic acid esters [104-108], and the response to local anaesthetic bases [109]. The reduction of methyl nicotinate-induced erythema by topical ibuprofen has also been correlated with the drug's concentration in the epidermis [110]. Topical retinoids have been shown to increase transepidermal water loss in a time and dose-dependent fashion [111,112]; however, the use of this effect for BE determination has not been confirmed nor recommended [113].

In summary, therefore, apart from the vasoconstrictor assay, which is clearly restricted, at this time, to TG, there are currently no non-invasive or minimally invasive techniques for the assessment of BA/BE of topically applied drugs that are acceptable to the regulatory bodies. For all other topically applied drugs, comparative clinical trials are the only approved means with which to establish BE. In an effort to address this situation and to provide viable alternatives for BE determination, significant efforts are being

directed to the dermatopharmacokinetic (DPK) approach, microdialysis and the use of *in vitro* experiments [114].

The DPK method uses tape-stripping to measure drug concentrations in the SC. The SC is collected by successive application and removal of adhesive tapes (Figure 10), which are subsequently extracted and analysed for the drug.

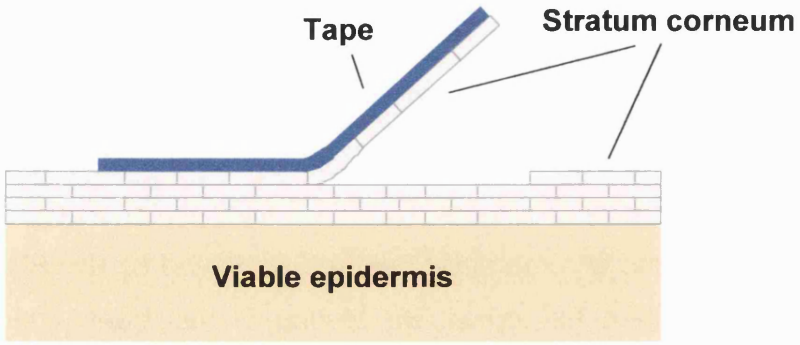


Figure 10: Schematic representation of the tape-stripping technique.

In theory, the DPK approach may be applied to all topical drugs. The principal assumption is that the amount of drug recovered from the SC, the usual barrier to percutaneous absorption, is directly correlated with the amount reaching the target cells. A recently published comparison of DPK and skin blanching response following topical application of triamcinolone acetonide and betamethasone dipropionate has validated this hypothesis [40,115]. The DPK concept, which evolved from a series of earlier studies reported by Rougier et al. [116], was introduced in a Draft Guidance from the FDA in 1998 [117]. Over the following 4 years, a number of concerns were raised with respect to the relevance of the approach to diseased skin, to the applicability of the method to drugs which have rather specific sites of action (for example, the hair follicle), and to the reproducibility and practicability of the technique. In 2002, a comparative study using tretinoin gels was performed in two laboratories and produced conflicting results [118-120]. This led, in turn, to withdrawal of the Draft Guidance. Subsequently, the FDA has been critically re-evaluating DPK, with a view to improving sensitivity, to reducing complexity, and to validating the approach sequentially for specific

drug classes. Other laboratories have also been contributing to this process and the value of improved DPK procedures has been demonstrated for the assessment of the BA of drugs whose site of action is the SC itself, such as: antifungal drugs [121-124], keratolytics [125-127], UVA/UVB filters [128-131], and antiseptics [132].

In principle, microdialysis allows continuous monitoring of the rate and extent of drug penetration into the skin. Via a probe (comprising a dialysis membrane) inserted into the dermis (Figure 11), the *in vivo* sampling of endogenous and exogenous substances in the extracellular fluid is possible. The technique can directly measure, therefore, drug levels within the skin and comes closest, as a result, to offering information about BA in the target tissue. Further, the method may be used when the skin's barrier is disrupted and has been shown to be applicable to measurements on diseased skin [133,134]. Typically, the microdialysis probe (~ 200 µm diameter) is situated 1-3 mm beneath the skin surface and is perfused with a physiological solution (perfusate) at a volume flow rate of ~ 5 µl/min. The molecular weight cut-off of the dialysis membrane is on the order of 20 kDa. The exchange of substances across the dialysis membrane occurs by passive diffusion and depends on the relevant concentration gradient. These attractive features must be balanced against a number of significant challenges. First, and foremost, the technique is invasive and is difficult to perform; although very little biological fluid is removed, the mere insertion of the microdialysis probe can cause transient inflammation and the local release of various biological factors (e.g., cytokines) [135-137]. Second, the duration of a microdialysis experiment is necessarily limited for practical reasons, and this creates a problem for slowly or poorly permeating substances. Relatedly, recoveries are typically low: that is, the concentration of analytes in the perfusate is so low that analysis becomes difficult; this is particularly true for lipophilic drugs, such as betamethasone 17-valerate, or drugs which are highly protein-bound, which do not distribute significantly into the aqueous perfusate in the microdialysis probe [138,139]. Finally, to relate the amount of analyte in the perfusate to its real concentration in the skin, it is necessary to use a so called "retrodialysis marker" (of similar physicochemical properties) to

calibrate the recovery efficiency [140-142]. Overall, while microdialysis is an alternative option, the outstanding challenges preclude, at the present time, its use for routine BA/BE evaluation.

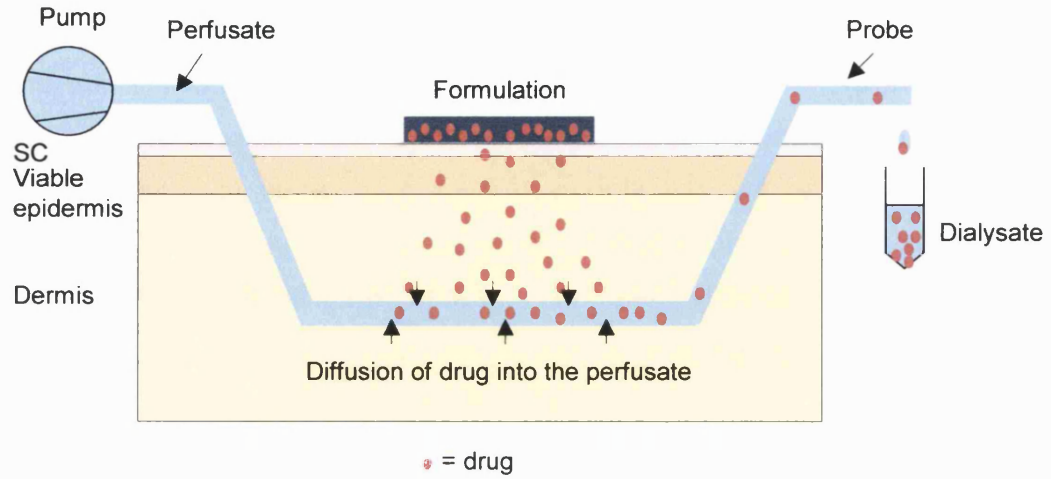


Figure 11: Schematic representation of the principle of the microdialysis. The probe is inserted in the dermis and is perfused at a flow rate controlled by the pump. Drug from the applied formulation permeates the SC and viable epidermis and eventually diffuses passively into the lumen of the membrane. The dialysate is sampled at fixed time intervals.

Finally, it is appropriate to describe briefly the value of *in vitro* procedures in the BA/BE evaluation of topical drug products. While the SUPAC-SS ("release test") procedure [143] is valuable as a tool for quality control, it is not appropriate for BE assessment except for very minor formulation changes. On the other hand, it may be said that *in vitro* skin permeation experiments have proven to be valuable guides in formulation development. However, the regulatory agencies (especially the FDA) have been reluctant to adopt such an approach for BA/BE determination. While there are reasonable concerns about the provision of human skin, of sufficient quality and quantity, for routine use, there are probably situations, particularly for drugs of balanced lipophilicity/hydrophilicity, in which an *in vitro* skin penetration comparison of formulations would be perfectly adequate to judge BE.

9. Conclusion

TG are an integral part of dermatological therapy. Despite years of use, however, the topical BA of these drugs remains difficult to assess and has rarely been properly optimised. While it is generally agreed that their BA is poor, a quantitative measure of this key parameter has proven elusive. *In vitro* experiments do provide flux and permeability values but, from a regulatory standpoint, this information is viewed as complementary rather than definitive and clinical trials are the 'gold standard' with which to assess efficacy. BE may, in the right circumstances, be determined using the vasoconstrictor assay, and the use of the chromameter to assess objectively skin blanching is an improvement in the application of this method. However, this determination of a pharmacological response remains some way from an absolute measurement of the amount of drug in the skin (at or near the target site) and the rate at which it arrived there. Consequently, much effort is presently focused on different approaches to estimate BA and BE of TG and other dermatological drugs. The dermatopharmacokinetic (tape-stripping) and microdialysis techniques are the most seriously considered at this time. Both have clear advantages and drawbacks, and it remains to be seen which one (if any) will emerge as a viable methodology to move forward and become a part of the regulatory process.

10. References

[1] Anderson R. L., Cassidy J. M., Variations in physical dimensions and chemical composition of human stratum corneum, J. Invest. Dermatol. 1973; 61:30-32

[2] Ponec M., Kempenaar J. A., De Kloet E. R., Corticoids and cultured human epidermal keratinocytes: specific intracellular binding and clinical efficacy, J. Invest. Dermatol. 1981; 76:211-214

[3] Ponec M., Polano M. K., Penetration of various corticosteroids through epidermis in vitro, Arch. Dermatol. Res. 1979; 265:101-104

[4] Scheuplein R. J., Blank I. H., Permeability of the skin, Physiol. Rev. 1971; 51:702-747

[5] Elias P. M., Epidermal lipids, barrier function, and desquamation, J. Invest. Dermatol. 1983; 80:44s-49s

[6] Harding C. R., The stratum corneum: structure and function in health and disease, Dermatol. Ther. 2004; 17:6-15

[7] Rawlings A. V., Harding C. R., Moisturization and skin barrier function, Dermatol. Ther. 2004; 17:43-48

[8] Swartzendruber D. C., Wertz P. W., Madison K. C., Downing D. T., Evidence that the corneocyte has a chemically bound lipid envelope, J. Invest. Dermatol. 1987; 88:709-713

[9] Marekov L. N., Steinert P. M., Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope, J. Biol. Chem. 1998; 273:17763-17770

[10] Kalbitz J., Neubert R., Wohlrab W., Modulation der Wirkstoffpenetration in die Haut, Pharmazie 1996; 51:619-637

[11] Elias P. M., Epidermal barrier function: intercellular lamellar lipid structures, origin, composition and metabolism, J. Controlled Release 1991; 15:199-208

[12] Landmann L., Die Permeabilitätsbarriere der Haut, Pharmazie in unserer Zeit 1991; 4:155-163

[13] Wertz P. W., Downing D. T. Stratum corneum: Biological and biochemical considerations. In: Hadgraft J, Guy RH, Editors. Transdermal Drug Delivery. New York: Marcel Dekker; 1989. p. 1-22.

[14] Potts R. O., Francoeur M. L., The influence of stratum corneum morphology on water permeability, J. Invest. Dermatol. 1991; 96:495-499

[15] Harada K., Murakami T., Yata N., Yamamoto S., Role of intercellular lipids in stratum corneum in the percutaneous permeation of drugs, J. Invest. Dermatol. 1992; 99:278-282

[16] Kasting G. B., Barai N. D., Wang T.-F., Nitsche J. M., Mobility of water in human stratum corneum, J. Pharm. Sci. 2003; 92:2326-2340

[17] Lavrijsen A. P. M., Bouwstra J. A., Gooris G. S., Weerheim A., Bodde H. E., Ponec M., Reduced skin barrier function parallels abnormal stratum corneum lipid organization in patients with lamellar ichthyosis, J. Invest. Dermatol. 1995; 105:619-624

[18] Hou S. Y. E., Mitra A. K., White S. H., Menon G. K., Ghadially R., Elias P. M., Membrane structures in normal and essential fatty acid-deficient stratum corneum: Characterization by ruthenium tetroxide staining and X-ray diffraction, J. Invest. Dermatol. 1991; 96:215-223

[19] Knutson K., Potts R. O., Guzek D. B., Golden G. M., McKie J. E., Lambert L. D., et al., Macro- and molecular physical - Chemical considerations in understanding drug transport in the stratum corneum, J. Controlled Release 1985; 2:67-87

[20] Rawlings A. V., Trends in stratum corneum research and the management of dry skin conditions, Int. J. Cosmet. Sci. 2003; 25:63-95

[21] Bouwstra J. A., Dubbelaar F. E. R., Gooris G. S., Weerheim A. M., Ponec M., The role of ceramide composition in the lipid organisation of the skin barrier, Biochim. et Biophys. Acta - Biomembranes 1999; 1419:127-136

[22] Bouwstra J. A., Cheng K., Gooris G. S., Weerheim A., Ponec M., The role of ceramides 1 and 2 in the stratum corneum lipid organisation, Biochim. Biophys. Acta 1996; 1300:177-186

[23] Bouwstra J. A., Gooris G. S., Dubbelaar F. E. R., Weerheim A. M., Ijzerman A. P., Ponec M., Role of ceramide 1 in the molecular organization of the stratum corneum lipids, J. Lipid Res. 1998; 39:186-196

[24] de Jager M. W., Gooris G. S., Ponec M., Bouwstra J. A., Lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behavior, J. Lipid Res. 2005; 46:2649-2656

[25] Grubauer G., Feingold K., Harris R., Elias P., Lipid content and lipid type as determinants of the epidermal permeability barrier, J. Lipid Res. 1989; 30:89-96

[26] Williams R. L., Riviere J. E., A biophysically based dermatopharmacokinetic compartment model for quantifying percutaneous penetration and absorption of topically applied agents. I. Theory, J. Pharm. Sci. 1995; 84:599-608

[27] Cross S. E., Magnusson B. M., Winckle G., Anissimov Y. G., Roberts M. S., Determination of the effect of lipophilicity on the in vitro permeability and tissue reservoir characteristics of topically applied solutes in human skin layers, J. Invest. Dermatol. 2003; 120:759-764

[28] Caron D., Queille-Roussel C., Shah V. P., Schaefer H., Correlation between the drug penetration and the blanching effect of topically applied hydrocortisone creams in human beings, J. Am. Acad. Dermatol. 1990; 23:458-462

[29] Marks R., Barlow J. W., Funder J. W., Steroid-induced vasoconstriction: Glucocorticoid antagonist studies, J. Clin. Endocrinol. Metab. 1982; 54:1075-1077

[30] Ponec M. Glucocorticoid Receptors. In: Korting HC, Maibach HI, editors. Topical Glucocorticoids with Increased Benefit/Risk Ratio. Basel: Karger; 1993. p. 20-28.

[31] Ponec M., Kempenaar J. A., Biphasic entry of glucocorticoids into cultured human skin keratinocytes and fibroblasts, Arch. Dermatol. Res. 1983; 275:334-344

[32] Baxter J. D., Minimizing the side effects of glucocorticoid therapy, Adv. Intern. Med. 1990; 35:173-194

[33] Rao G. S., Mode of entry of steroid and thyroid hormones into cells, Mol. Cell. Endocrinol. 1981; 21:97-108

[34] Hughes J., Rustin M., Corticosteroids, Clin. Dermatol. 1997; 15:715-721

[35] Oakley R. H., Cidlowski J. A., Homologous down regulation of the glucocorticoid receptor: The molecular machinery, Crit. Rev. Eukaryot. Gene Expr. 1993; 3:63-88

[36] Scheinman R. I., Cogswell P. C., Lofquist A. K., Baldwin A. S. J., Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids, Science 1995; 270:283-286

[37] Auphan N., DiDonato J. A., Rosette C., Helmberg A., Karin M., Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis, Science 1995; 270:286-290

[38] Almawi W., Lipman M., Stevens A., Zanker B., Hadro E., Strom T., Abrogation of glucocorticoid-mediated inhibition of T cell proliferation by the synergistic action of IL-1, IL-6, and IFN-gamma, J. Immunol. 1991; 146:3523-3527

[39] Lange K., Kleuser B., Gysler A., Bader M., Maia C., Scheidereit C., et al., Cutaneous inflammation and proliferation in vitro: differential effects and mode of action of topical glucocorticoids, Skin Pharmacol. Appl. Skin Physiol. 2000; 13:93-103

[40] Pershing L. K., Silver B. S., Krueger G. G., Shah V. P., Skelley J. P., Feasibility of measuring the bioavailability of topical betamethasone dipropionate in commercial formulations using drug content in skin and a skin blanching assay, Pharm. Res. 1992; 9:45-51

[41] McKenzie A. W., Atkinson R. M., Topical activities of betamethasone esters in man, Arch. Dermatol. 1964; 89:741-746

[42] Stoughton R. B., Are generic formulations equivalent to trade name topical glucocorticoids?, Arch. Dermatol. 1987; 123:1312-1314

[43] Pershing L. K., Lambert L. D., Wright E. D., Shah V. P., Williams R. L., Topical 0.050% betamethasone dipropionate pharmacokinetic and pharmacodynamic dose-response studies in humans, Arch. Dermatol. 1994; 130:740-747

[44] Noon J. P., Evans C. E., Haynes W. G., Webb D. J., Walker R. B., A comparison of techniques to assess skin blanching following the topical application of glucocorticoids, Br. J. Dermatol. 1996; 134:837-842

[45] Korting H. C., Kerscher M. J., Schäfer-Korting M., Topical glucocorticoids with improved benefit/risk ratio: do they exist?, J. Am. Acad. Dermatol. 1992; 27:87-92

[46] Sheu H.-M., Lee J. Y.-Y., Chai C.-Y., Kuo K.-W., Depletion of stratum corneum intercellular lipid lamellae and barrier function abnormalities after long-term topical corticosteroids, Br. J. Dermatol. 1997; 136:884-890

[47] Sheu H.-M., Tai C.-L., Kuo K.-W., Modulation of epidermal terminal differentiation in patients after long-term topical corticosteroids, J. Dermatol. 1991; 18:454-464

[48] Sheu H. M., Lee J. Y., Kuo K. W., Tsai J. C., Permeability barrier abnormality of hairless mouse epidermis after topical corticosteroid: Characterization of stratum corneum lipids by ruthenium tetroxide staining and high-performance thin-layer chromatography, J. Dermatol. 1998; 25:281-289

[49] Woodbury R., Kligman A. M., The hairless mouse model for assaying the atrophogenicity of topical corticosteroids, Acta Derm. Venereol. 1992; 72:403-406

[50] Kao J. S., Fluhr J. W., Man M.-Q., Fowler A. J., Hachem J.-P., Crumrine D., et al., Short-term glucocorticoid treatment compromises both permeability barrier homeostasis and stratum corneum integrity: Inhibition of epidermal lipid synthesis account for functional abnormalities, J. Invest. Dermatol. 2003; 120:456-464

[51] Kerscher M. J., Korting H. C., Comparative atrophogenicity potential of medium and highly potent topical glucocorticoids in cream and ointment according to ultrasound analysis, Skin Pharmacol. 1992; 5:77-80

[52] Lebwohl M., Ali S., Treatment of psoriasis. Part 1. Topical therapy and phototherapy, J. Am. Acad. Dermatol. 2001; 45:487-498

[53] Schäfer-Korting M., Korting H. C., Kerscher M. J., Lenhard S., Prednicarbate activity and benefit/risk ratio in relation to other topical glucocorticoids, Clin. Pharmacol. Ther. 1993; 54:448-456

[54] Mori M., Pimpinelli N., Giannotti B., Topical corticosteroids and unwanted local effects. Improving the benefit/risk ratio, Drug Saf. 1994; 10:406-412

[55] Kecskes A., Jahn P., Wendt H., Lange L., Kuhlmann R. K., Dose-response relationship of topically applied methylprednisolone aceponate (MPA) in healthy volunteers, Eur. J. Clin. Pharmacol. 1992; 43:157-159

[56] Ruzicka T., Methylprednisolone aceponate* in eczema and other inflammatory skin disorders - a clinical update, Int. J. Clin. Pract. 2006; 60:85-92

[57] Hoffmann K., Auer T., Stücker M., Hoffmann A., Altmeyer P., Comparison of Skin Atrophy and vasoconstriction due to mometasone furoate, methylprednisolone and hydrocortisone, J. Eur. Acad. Dermatol. Venereol. 1998; 10:137-142

[58] Korting H. C., Unholzer A., Schäfer-Korting M., Tausch I., Gassmueller J., Nietsch K.-H., Different skin thinning potential of equipotent medium-strength glucocorticoids, Skin Pharmacol. Appl. Skin Physiol. 2002; 15:85-91

[59] Gysler A., Kleuser B., Sippl W., Lange K., Korting H. C., Höltje H.-D., et al., Skin penetration and metabolism of topical glucocorticoids in reconstructed epidermis and in excised human skin, Pharm. Res. 1999; 16:1386-1391

[60] Lange K., Gysler A., Bader M., Kleuser B., Korting H.-C., Schaefer-Korting M., Prednicarbate versus conventional topical glucocorticoids: Pharmacodynamic characterization in vitro, Pharm. Res. 1997; 14:1744-1749

[61] Gysler A., Lange K., Korting H. C., Schäfer-Korting M., Prednicarbate biotransformation in human foreskin keratinocytes and fibroblasts, Pharm. Res. 1997; 14:793-797

[62] Spencer C. M., Wiseman L. R., Topical fluticasone propionate - A review of its pharmacological properties and therapeutic use in the treatment of dermatological disorders, Biodrugs 1997; 7:318-334

[63] Federman D. G., Froelich C. W., Kirsner R. S., Topical psoriasis therapy, Am. Fam. Physician. 1999; 59:957-962

[64] Pershing L. K., Corlett J. L., Lambert L. D., Poncelet C. E., Circadian activity of topical 0.05% betamethasone dipropionate in human skin in vivo, J. Invest. Dermatol. 1994; 102:734-739

[65] National Psoriasis Foundation P. O., Steroids, 1998

[66] British National Formulary. London: British Medical Association and the Royal Pharmaceutical Society of Great Britain; 2004

[67] Brazzini B., Pimpinelli N., New and established topical corticosteroids in dermatology, Am. J. Clin. Dermatol. 2002; 3:47-58

[68] Lippold B. C., Teubner A., Biopharmazeutische Qualität von Arzneiformen, insbesondere für lokale Anwendung, abgeleitet aus Wirkungsmessungen, Pharm. Ind. 1981; 43:71-73

[69] Katz M., Poulsen B. J., Corticoid, vehicle, and skin interaction in percutaneous absorption, J. Soc. Cosmet. Chem. 1972; 23:565-590

[70] Katz M., Shaikh Z. I., Percutaneous corticosteroid absorption correlated to partition coefficient, J. Pharm. Sci. 1965; 54:591-594

[71] Bach M., Lippold B. C., Influence of penetration enhancers on the blanching intensity of betamethasone 17-benzoate, Int. J. Pharm. 1998; 168:97-108

[72] Lippold B. C., Schneemann H., The Influence of vehicles on the local bioavailability of betamethasone-17-benzoate from solution- and suspension-type ointments, Int. J. Pharm. 1984; 22:31-43

[73] Ayres J. W., Hooper G., Assessment of the skin penetration properties of different carrier vehicles for topically applied cortisol, Br. J. Dermatol. 1978; 99:307-317

[74] Poulsen J., Rorsman H., Ranking of glucocorticoid creams and ointments, Acta Derm. Venereol. 1980; 60:57-62

[75] Cornell R. C., Stoughton R. B., Correlation of the vasoconstriction assay and clinical activity in psoriasis, Arch. Dermatol. 1985; 121:63-67

[76] Stoughton R. B., Bioassay system for formulations of topically applied glucocorticosteroids, Arch. Dermatol. 1972; 106:825-827

[77] Stein L., Clinical studies of a new vehicle formulation for topical corticosteroids in the treatment of psoriasis, J. Am. Acad. Dermatol. 2005; 53:S39-S49

[78] Franz T. J., Parsell D. A., Halualani R. M., Hannigan J. F., Kalbach J. P., Ma, et al., Betamethasone valerate foam 0.12%: a novel vehicle with enhanced delivery and efficacy, Int. J. Dermatol. 1999; 38:628-632

[79] Woodford R., Barry B. W., Bioavailability and activity of betamethasone 17-benzoate in gel and cream formulations: Comparison with proprietary topical corticosteroid preparations in the vasoconstrictor assay, Curr. Ther. Res. 1974; 16:338-345

[80] Lippold B. C., Kutane Resorption - Möglichkeiten, Modelle, Beeinflussung, Acta Pharm. Technol. 1981; 27:1-9

[81] Poulsen B. J., Diffusion of drugs from topical vehicles: an analysis of vehicle effects, Adv. Biol. Skin. 1972; 12:495-509

[82] Gao H. Y., Li Wan Po A., Topical formulations of fluocinolone acetonide, Eur. J. Clin. Pharmacol. 1994; 46:71-75

[83] Ryatt K. S., Feather J. W., Mehta A., Dawson J. B., Cotterill J. A., Swallow R., The stability and blanching efficacy of betamethasone-17-valerate in emulsifying ointment, Br. J. Dermatol. 1982; 107:71-76

[84] Refai H., Müller-Goymann C. C., The influence of dilution of topical semisolid preparations on hydrocortisone permeation through excised human stratum corneum, Eur. J. Pharm. Biopharm. 2002; 54:143-150

[85] National Archives and Records Administration, Code of federal regulations (title 21) Food and Drugs, Revised 2001 Apr 1, Available from URL: http://www.access.gpo.gov/cgi-bin/cfrassemble.-cgi?title=200121

[86] Shah V. P., Flynn G. L., Yacobi A., Maibach H. I., Williams R. L., et al., Bioequivalence of topical dermatological dosage forms-methods of evaluation of bioequivalence, Pharm. Res. 1998; 15:167-171

[87] Guidance for Industry: Topical dermatologic corticosteroids: In vivo bioequivalence, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1995

[88] McKenzie A. W., Stoughton R. B., A method for comparing percutaneous absorption of steroids, Arch. Dermatol. 1962; 86:608-610

[89] McKenzie A. W., Percutaneous absorption of steroids, Arch. Dermatol. 1962; 86:611-614

[90] Haigh J. M., Kanfer I., Assessment of topical corticosteroid preparations: The human skin blanching assay, Int. J. Pharm. 1984; 19:245-262

[91] Gibson J. R., Kirsch J. M., Darley C. R., Harvey S. G., Burke C. A., Hanson M. E., An assessment of the relationship between vasoconstrictor assay findings, clinical efficacy and skin thinning effects of a variety of undiluted and diluted corticosteroid preparations, Br. J. Dermatol. 1984; 111:204-212

[92] Barry B. W., Woodford R., Activity and bioavailability of topical steroids. In vivo/in vitro correlations for the vasoconstrictor test, J. Clin. Pharm. 1978; 3:43-65

[93] Jackson D. B., Thompson C., McCormack J. R., Guin J. D., Bioequivalence (bioavailability) of generic topical corticosteroids, J. Am. Acad. Dermatol. 1989; 20:791-796

[94] Demana P. H., Smith E. W., Walker R. B., Haigh J. M., Kanfer I., Evaluation of the proposed FDA pilot dose response methodology for topical corticosteroid bioequivalence testing, Pharm. Res. 1997; 14:303-308

[95] Leopold C. S., Pharmacokinetic analysis of the FDA guidance for industry - "Topical dermatologic corticosteroids: in vivo bioequivalence", Eur. J. Pharm. Biopharm. 2003; 56:53-58

[96] Leopold C. S., Quantification of depletion in solution-type topical preparations in vivo, J. Cosmet. Sci. 1998; 49:165-174

[97] Schwarb F. P., Smith E. W., Haigh J. M., Surber C., Analysis of chromameter results obtained from corticosteroid-induced skin blanching assay: comparison of visual and chromameter data, Eur. J. Pharm. Biopharm. 1999; 47:261-267

[98] Montenegro L., Ademola J. I., Bonina F. P., Maibach H. I., Effect of application time of betamethasone-17-valerate 0.1% cream on skin blanching and stratum corneum drug concentration, Int. J. Pharm. 1996; 140:51-60

[99] Pershing L. K., New approaches to assess topical corticosteroid bioequivalence: Pharmacokinetic evaluation, Int. J. Dermatol. 1992; 31:14-20

[100] Clarys P., Alewaeters K., Lambrecht R., Barel A. O., Skin color measurement: comparison between three instruments: the Chromameter, the DermaSpectrometer and the Mexameter, Skin Res. Technol. 2000; 6:230-238

[101] Smith E. W., Haigh J. M., Surber C., Quantification of corticosteroid-induced skin vasoconstriction: Visual ranking, chromameter measurement or digital imaging analysis, Dermatology 2002; 205:3-10

[102] Haigh J. M., Purdon C. H., Smith E. W., Maibach H. I. Methods for the Assessment of Topical Corticosteroid-Induced Skin Blanching. In: Bronaugh RL, Maibach HI, Editors. Topical Absorption of Dermatological Products. New York: Marcel Dekker, Inc.; 2002. p. 275-282

[103] Walker R. B., Haigh J. M., Smith E. W. Application of the Minolta chromameter to the assessment of corticosteroid-induced skin blanching. In: Schwindt DA, Maibach HI, Editors. Cutaneous Biometrics. New York: Kluwer Academic/Plenum; 2000. p. 295-305.

[104] Chan S. Y., Po A. L. W., Quantitative assessment of non-steroidal anti-inflammatory topical products in nicotinate-induced erythema using tristimulus colour analysis, Int. J. Pharm. 1992; 83:73-86

[105] Lippold B. C., Reimann H., Wirkungsbeeinflussung bei Lösungssalben durch Vehikel am Beispiel von Methylnicotinat, Acta Pharm. Technol. 1989; 35:136-142

[106] Chan S. Y., Li Wan Po A., Quantitative evaluation of drug-induced erythema by using a tristimulus colour analyzer: experimental design and data analysis, Skin Pharmacol. 1993; 6:298-312

[107] Leopold C. S., Maibach H. I., Effect of lipophilic vehicles on in vivo skin penetration of methyl nicotinate in different races, Int. J. Pharm. 1996; 139:161-167

[108] Mueller B., Kasper M., Surber C., Imanidis G., Permeation, metabolism and site of action concentration of nicotinic acid

derivatives in human skin: Correlation with topical pharmacological effect, Eur. J. Pharm. Sci. 2003; 20:181-195

[109] Leopold C., Maibach H. I., Percutaneous penetration of local anesthetic bases: Pharmacodynamic measurement, J. Invest. Dermatol. 1999; 113:304-307

[110] Treffel P., Gabard B., Feasibility of measuring the bioavailability of topical ibuprofen in commercial formulations using drug content in epidermis and a methyl nicotinate skin inflammation assay, Skin Pharmacol. 1993; 6:268-275

[111] Tagami H., Tadaki T., Obata M., Koyama J., Functional assessment of the stratum corneum under the influence of oral aromatic retinoid (etretinate) in guinea-pigs and humans. Comparison with topical retinoic acid treatment, Br. J. Dermatol. 1992; 127:470-475

[112] Verschoore M., Poncet M., Czernielewski J., Sorba V., Clucas A., Adapalene 0.1% gel has low skin-irritation potential, J. Am. Acad. Dermatol. 1997; 36:S104-109

[113] Fluhr J. W., Vienne M. P., Lauze C., Dupuy P., Gehring W., Gloor M., Tolerance profile of retinol, retinaldehyde and retinoic acid under maximized and long-term clinical conditions, Dermatology 1999; 199 Suppl. 1:57-60

[114] Shah V. P., Topical drug products - microdialysis: regulatory perspectives, Int. J. Clin. Pharmacol. Ther. 2004; 42:379-381

[115] Pershing L. K., Bakhtian S., Poncelet C. E., Corlett J. L., Shah V. P., Comparison of skin stripping, in vitro release, and skin blanching response methods to measure dose response and similarity of triamcinolone acetonide cream strengths from two manufactured sources, J. Pharm. Sci. 2002; 91:1312-1323

[116] Rougier A., Dupuis D., Lotte C., Roguet R., Schaefer H., In vivo correlation between stratum corneum reservoir function and percutaneous absorption., J. Invest. Dermatol. 1983; 81:275-278

[117] Guidance for Industry: Topical dermatologic drug product NDAs and ANDAs-in vivo bioavailability, bioequivalence, in vitro release, and associated studies, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1998

[118] Conner D. P. Differences in DPK Methods. http://www.fda.gov /ohrms/dockets/ac/01/slides/3804s2_01_conner/index.htm, Advisory Committee for Pharmaceutical Sciences Meeting, Center for Drug Evaluation and Research (CDER), Food and Drug Administration (FDA), Rockville, MD; November 29, 2001

[119] Franz T. J. Study #1, Avita Gel 0.025% vs Retin-A Gel 0.025%, Advisory Committee for Pharmaceutical Sciences Meeting, Center for Drug Evaluation and Research (CDER), Food and Drug Administration (FDA), Rockville, MD; November 29, 2001

[120] Pershing L. K. Bioequivalence assessment of three 0.025% tretinoin gel products: Dermatopharmacokinetic vs. clinical trial methods. Advisory Committee for Pharmaceutical Sciences Meeting, Center for Drug Evaluation and Research (CDER), Food and Drug Administration (FDA), Rockville, MD; November 29, 2001

[121] Alberti I., Kalia Y. N., Naik A., Bonny J.-D., Guy R. H., In vivo assessment of enhanced topical delivery of terbinafine to human stratum corneum, J. Controlled Release 2001; 71:319-327

[122] Alberti I., Kalia Y. N., Naik A., Bonny J.-D., Guy R. H., Effect of ethanol and isopropyl myristate on the availability of topical terbinafine in human stratum corneum, in vivo, Int. J. Pharm. 2001; 219:11-19

[123] Alberti I., Kalia Y. N., Naik A., Guy R. H., Assessment and prediction of the cutaneous bioavailability of topical terbinafine, in vivo, in man, Pharm. Res. 2001; 18:1472-1475

[124] Pershing L. K., Nelson J. L., Corlett J. L., Shrivastava S. P., Hare D. B., Shah V. P., Assessment of dermatopharmacokinetic approach in the bioequivalence determination of topical tretinoin gel products, J. Am. Acad. Dermatol. 2003; 48:740-751

[125] Bashir S. J., Dreher F., Chew A. L., Zhai H., Levin C., Stern R., et al., Cutaneous bioassay of salicylic acid as a keratolytic, Int. J. Pharm. 2005; 292:187-194

[126] Schwarb F. P., Gabard B., Rufli T., Surber C., Percutaneous absorption of salicylic acid in man after topical administration of three different formulations, Dermatology 1999; 198:44-51

[127] Tsai J.-C., Chuang S.-A., Hsu M.-Y., Sheu H.-M., Distribution of salicylic acid in human stratum corneum following topical application in vivo: a comparison of six different formulations, Int. J. Pharm. 1999; 188:145-153

[128] Jacobi U., Weigmann H.-J., Baumann M., Reiche A.-I., Sterry W., Lademann J., Lateral spreading of topically applied UV filter substances investigated by tape stripping, Skin Pharmacol. Physiol. 2004; 17:17-22

[129] Teichmann A., Jacobi U., Weigmann H. J., Sterry W., Lademann J., Reservoir function of the stratum corneum: development of an in vivo method to quantitatively determine the stratum corneum reservoir for topically applied substances, Skin Pharmacol. Physiol. 2005; 18:75-80

[130] Gamer A. O., Leibold E., van Ravenzwaay B., The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin, Toxicol. in Vitro 2005; 20:301-307

[131] Wissing S. A., Müller R. H., Solid lipid nanoparticles as carrier for sunscreens: In vitro release and in vivo skin penetration, J. Controlled Release 2002; 81:225-233

[132] Lboutounne H., Chaulet J.-F., Ploton C., Falson F., Pirot F., Sustained ex vivo skin antiseptic activity of chlorhexidine in poly([epsilon]-caprolactone) nanocapsule encapsulated form and as a digluconate, J. Controlled Release 2002; 82:319-334

[133] Benfeldt E., Serup J., Effect of barrier perturbation on cutaneous penetration of salicylic acid in hairless rats: in vivo pharmacokinetics using microdialysis and non-invasive quantification of barrier function, Arch. Dermatol. Res. 1999; 291:517-526

[134] Benfeldt E., Serup J., Menne T., Effect of barrier perturbation on cutaneous salicylic acid penetration in human skin: in vivo pharmacokinetics using microdialysis and non-invasive quantification of barrier function, Br. J. Dermatol. 1999; 140:739-748

[135] Groth L., Serup J., Cutaneous microdialysis in man: effects of needle insertion trauma and anaesthesia on skin perfusion, erythema and skin thickness, Acta Derm. Venereol. 1998; 78:5-9

[136] Sjogren F., Svensson C., Anderson C., Technical prerequisites for in vivo microdialysis determination of interleukin-6 in human dermis, Br. J. Dermatol. 2002; 146:375-382

[137] Linden M., Andersson T., Wardell K., Anderson C., Is vascular reactivity in skin predictable?, Skin Res. Technol. 2000; 6:27-30

[138] Benfeldt E., Groth L., Feasibility of measuring lipophilic or protein-bound drugs in the dermis by in vivo microdialysis after topical or systemic drug administration, Acta Derm. Venereol. 1998; 78:274-278

[139] Groth L., Jorgensen A., In vitro microdialysis of hydrophilic and lipophilic compounds, Analytica Chimica Acta 1997; 355:75-83

[140] Simonsen L., Jorgensen A., Benfeldt E., Groth L., Differentiated in vivo skin penetration of salicylic compounds in hairless rats measured by cutaneous microdialysis, Eur. J. Pharm. Sci. 2004; 21:379-388

[141] Schwalbe O., Buerger C., Plock N., Joukhadar C., Kloft C., Urea as an endogenous surrogate in human microdialysis to determine relative recovery of drugs: Analytics and applications, J. Pharm. Biomed. Anal. 2006; 41:233-239

[142] Mathy F.-X., Preat V., Verbeeck R. K., Validation of subcutaneous microdialysis sampling for pharmacokinetic studies of flurbiprofen in the rat, J. Pharm. Sci. 2001; 90:1897-1906

[143] Guidance for Industry: SUPAC-SS Nonsterile semisolid dosage forms, scale-up and post approval changes: Chemistry, manufacturing and controls; in vitro release testing and in vivo bioequivalence documentation, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1997

CHAPTER 2

Topical application of betamethasone 17-valerate formulations: Tape-stripping versus pharmacodynamic response

Topical application of betamethasone 17-valerate formulations: Tape-stripping versus pharmacodynamic response

Sandra Wiedersberg[1], Aarti Naik[2], Claudia S. Leopold[3], Richard H. Guy[1]

[1]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK.
[2]School of Pharmaceutical Sciences, University of Geneva, 30 quai E. Ansermet, CH-1211 Geneva 4, Switzerland.
[3]Institute for Pharmacy, Department of Pharmaceutical Technology, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany.

Research paper to be submitted

Abstract

Objective: To compare the *in vivo* bioavailability profiles of two formulations of betamethasone 17-valerate (BMV) assessed using the dermatopharmacokinetic (DPK) approach and the vasoconstrictor assay.

Methods: BMV was formulated either in a medium chain triglyceride gel (MCT) or in a brand microemulsion (ME), at 80 % of its saturation level. Drug uptake into and elimination from the stratum corneum (SC) were assessed by the DPK approach using tape-stripping. The skin blanching response was quantified during 24 hours after a 2 and 6 hour drug application.

Results: With the vasoconstrictor assay, no difference in the blanching response between the two formulations was observed; however, there was a statistical difference in the amount of BMV taken up into the SC between the two formulations as measured by the DPK approach. BMV delivery into the SC was 5-fold higher from the ME than that from the MCT formulation.

Conclusion: The results of the vasoconstrictor assay suggest that the blanching response had been saturated, preventing any differences between the two formulations to be observed. In contrast, the DPK method, which was

quite reproducible, distinguished clearly between the delivery performance of the two vehicles.

1. Introduction

The efficiency of topical delivery is notoriously inefficient, with typical bioavailabilities of only a few percent of the applied dose. Rational development of better formulations requires better creativity and better methods with which to quantify bioavailability (BA) and bioequivalence (BE) of drug delivery to a target in the skin. Topical BA/BE evaluation typically requires clinical trials, which are invasive, relatively insensitive, time-consuming and costly. In the case of topical glucocorticoids (TG), however, BA/BE can be assessed using the vasoconstrictor assay. This non-invasive method relies on the pharmacodynamic response of TG in the upper layers of the skin that causes visual and quantifiable blanching due to vasoconstriction and, in 1995, the U.S. Food and Drug Administration (FDA) published a Guidance document for TG BE testing [1]. This Guidance consists of two distinct *in vivo* components: a pilot study and a pivotal test. The pilot study is carried out to explore the dose duration-response relationship of a reference listed drug (RLD) and to determine the appropriate dose duration for use in the pivotal test. The pivotal test then compares the *in vivo* response of the test product with that of the RLD to document whether BE has, or has not, been found.

Apart from TG, for all other topical drugs, however, the vasoconstrictor assay is not useful to document BA/BE and at the moment, clinical trials are the only option. In 1998, the FDA proposed an alternative, potentially more generally applicable, technique instead: the dermatopharmacokinetic approach (DPK), analogous to the pharmacokinetic method of oral drug BA/BE assessment [2,3]. The DPK approach, using tape-stripping, evaluates topically applied drug levels in the outermost layer of the skin, the stratum corneum (SC), as a function of time post-application and post-removal of the formulation. The Draft Guidance [2] allows the assessment of both drug uptake into and drug elimination from the SC. At specific times, layers of the SC are sequentially removed at the treated site with adhesive tapes and the total amount of drug is subsequently analysed therein. From the DPK profile of drug mass in the SC as a function of time, pharmacokinetic parameters such as the area under the curve (AUC), the maximum amount drug in the

tape-strips (A_{max}) and the time (T_{max}) at which A_{max} is observed are deduced and used to characterize the local BA.

It is assumed that SC drug levels are directly related to those in the epidermis and/or dermis, as the SC is typically the rate-determining barrier to percutaneous absorption. In other words, it is hypothesized that the rate and extent of drug disposition in the SC will reflect that achieved at target sites which are further into the skin. While the DPK Guidance has been withdrawn, and the details of the methodology are being reconsidered, there have been experiments using commercially available TG products in which the drug uptake phase into the SC using tape-stripping have been favourably compared with the pharmacodynamic response [4-7]. On the other hand, there has been criticism made of the DPK procedure used in these earlier experiments, especially with regard to the need for better quantification and control of the amount of SC removed and subsequently extracted, and certain modifications in the procedure have been recommended. However, apart from studies with tretinoin [8-10], there have been no DPK experiments with TG, investigating both the uptake and elimination phases.

The goal of this work, therefore, was to compare the topical BA profiles of two formulations of betamethasone 17-valerate obtained using the methods of the vasoconstrictor assay and of the DPK approach as specified in the Draft Guidance [2]. Drug concentrations in the vehicles compared were adjusted to the same thermodynamic activity. Pharmacokinetic (model-based) parameters of drug disposition in the SC, together with the 'classic' metrics of (i) the area under the curve, (ii) the magnitude of the maximum delivery/response, and (iii) the time required to reach this maximum, were determined and used to characterize local BA. Given the typical dosing regimen of the drug, the longest treatment time to characterize the uptake phase in the SC was chosen to be 6 hours. For the vasoconstrictor assay, an additional dose duration of 2 hours was investigated. The 'reference' formulation comprised medium chain triglycerides, which were not expected to exert specific vehicle effects [11]. The 'test' vehicle was a commercial microemulsion that might be expected to increase drug delivery to the skin.

2. Materials and methods

2.1. Preparation of the formulations

Betamethasone 17-valerate (BMV) (Crystal Pharma, Boecillo, Spain) was dissolved in the reference vehicle consisting of medium chain triglycerides (MCT) (Mygliol 812 N, Synopharm, Barsbüttel, Germany) and in the test microemulsion Mikro 100® (ME) (Sebapharma, Boppard, Germany). The components of the ME were aqua, polysorbate 20, polyglyceryl-6 dioleate, ethylhexyl cocoate, PEG-8 caprylic/capric glycerides, alcohol denat., tocopheryl acetate, ectoin, panthenol, centella asiatica, ethoxydiglycol oleate, sodium lactate, parfum and phenoxyethanol. The BMV concentration was adjusted to 80 % of the saturation level (Table 1) to ensure equivalent thermodynamic activity. The saturation levels of BMV in MCT and ME were determined by stirring a suspension of drug substance in the vehicles at 32 °C until equilibrium was attained (about 72 hours). The samples were centrifuged, appropriately diluted and analysed by HPLC (see method below). The experiments were performed in triplicate. To avoid spreading of the formulations on the skin, MCT and ME were gelled, with 15 % (w/w) polypropylene and 10 % (w/w) Aerosil® 200 (Sigma-Aldrich, Steinheim, Germany), respectively.

Table 1: Saturation levels of BMV at 32 °C in two vehicles. Mean ± SD, n = 3.

Vehicle	Saturation level [mg/ml]
MCT	2.1 ± 0.2
ME	11.7 ± 0.4

2.2. DPK study

The DPK study was carried out according to the FDA Draft Guidance [2]. Six human volunteers (females, aged 25 - 32 years) participated in the study, which was approved by the Commission d'Ethique, Département des Neurosciences cliniques et Dermatologie, Hôpitaux Universitaires de Genève. All subjects were in good general health and had no history of dermatological disease. The same infinite volume (250 µl) of each

formulation was placed in a 1.2 cm diameter Hill Top Chamber® (Hill Top Research Inc., Cincinnati, OH, USA) and affixed via an adhesive tape to the volar aspect of the forearm. A maximum of six chambers, three with the MCT formulation and three with the ME, were applied to each forearm (Figure 1). For drug uptake, the formulations were applied to the left forearm and the SC samples were collected from each site immediately after removal of the chambers at 2, 4 and 6 hours. To assess drug elimination, the formulations were applied to the right forearm, and maintained in place for 6 hours. All formulations were then removed (using dry paper swabs) and SC samples were taken after a further 2, 6 and 24 hours.

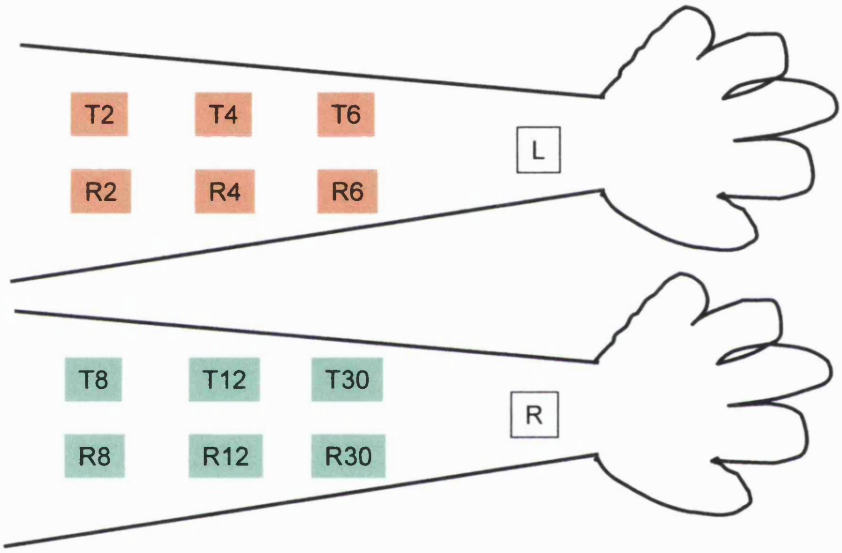


Figure 1: Application scheme. T = test vehicle (ME), R = reference vehicle (MCT). Uptake phase 2 to 6 hours ■; elimination phase 8 to 30 hours ■.

SC stripping used Scotch Book Tape (3M, St. Paul, MN, USA). The SC sampling site was delimited by a template, which was centred over the drug application site. The tape (2 x 2 cm) was applied over the template, pressed uniformly and removed. Each treated skin site was initially tape-stripped 12 times; additional tape-strips were taken, if necessary, until the value of transepidermal water loss (TEWL) was 4-fold greater then the pre-stripping value measured at an adjacent skin site and untreated with either formulation. Periodic measurements of TEWL (Evaporimeter EP1,

Servomed, Stockholm, Sweden), before and after the stripping process, were performed. A 4-fold increase in the TEWL value should ensure that at least 75 % of the SC was removed at each skin site [12]. The first tape-strip was discarded to avoid potential residual drug contamination. The drug on the remaining tape-strips was subsequently extracted and analysed by HPLC (see method below). The total amount of BMV recovered from the tape-strips was expressed in micrograms per square centimetre (µg/cm²).

2.3. Extraction and HPLC analysis of BMV

The tapes were extracted, in groups of 5 or fewer, by shaking overnight with 2.0 ml of 60:40 (v/v) acetonitrile/water (Sigma-Aldrich, Steinheim, Germany). BMV in the extracted samples was then quantified by HPLC (Dionex, Sunnyvale, CA, USA) with UV detection at 239 nm using a Lichrospher® 100 RP-18 (4 x 125 mm) column (Merck, Darmstadt, Germany). The mobile phase consisted of degassed acetonitrile - distilled water - triethylamine - phosphoric acid (60:40:0.1:0.05 v/v); the flow rate was 1 ml/min with a 50-µl sample loop. The retention time of BMV was 3.4 minutes.

2.4. Vasoconstrictor assay

The vasoconstrictor assay was carried out according to the pilot study (staggered application with synchronized removal) described in the FDA Guidance, "Topical Dermatologic Corticosteroids: *In vivo* Bioequivalence" [1]. Six healthy volunteers (3 male, 3 female, aged 21 - 55 years) from whom informed consent was obtained, participated in the study, which was approved by the Ethics Committee of the University of Leipzig. Once again, 250 µl of each formulation were applied via a 1.2 cm diameter Hill Top Chamber® to the ventral forearm. In addition, a drug-free vehicle control was applied. Dose durations of 2 and 6 hours were chosen. The skin blanching response was assessed with a chromameter (CR-300, Minolta, Ahrensburg, Germany) using the a-scale values at 0, 2, 4, 6, 19 and 24 hours after formulation removal (Figure 2). The instrument was calibrated using a white plate immediately before use. Baseline readings were taken at all sites prior to the application of the formulations. The a-scale readings for each drug

application site were adjusted for the baseline value and the control and expressed as the change in this parameter (Δa).

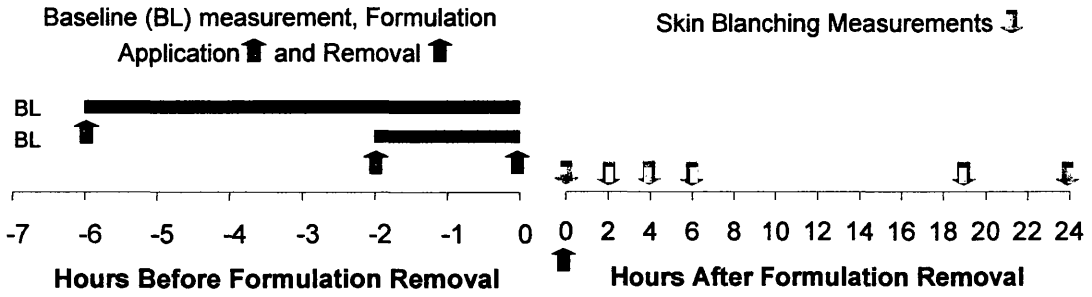


Figure 2: Staggered application with synchronized removal: Schematic representation of the pilot study protocol [1].

2.5 Pharmacokinetic analysis

For assessment of the BA of BMV delivered from the two formulations, the area under the curve (AUC), the maximum amount of BMV in the tape-strips (A_{max}), and the time point at which this maximum was attained (T_{max}), were determined from the DPK profiles. From the blanching response profiles (Δa versus time after formulation removal), the area above the response curve (AARC), the maximum blanching response (Δa_{max}), and T_{max} were estimated. The AUCs and AARCs were calculated using the trapezoidal method.

For both the DPK method and for the vasoconstrictor assay (VA), the increased BA of the test vehicle (ME) relative to that of the reference (MCT) was expressed as an enhancement factor EF according to the following equations:

$$EF_{DPK} = \frac{AUC_{ME}}{AUC_{MCT}} \qquad \text{Eq. 1}$$

$$EF_{VA} = \frac{AARC_{ME}}{AARC_{MCT}} \qquad \text{Eq. 2}$$

The DPK profiles of SC levels of BMV as a function of time were modelled assuming that the infinite doses applied provided an essentially zero-order delivery of drug into the measurement 'compartment' from which a first-order clearance subsequently occurred:

$$(BMV)_{surface} \xrightarrow[t \leq 6\ h]{k_0} (BMV)_{SC} \xrightarrow{k_e} (BMV)_{e/d}$$

where k_0 and k_e were zero-order input and first-order elimination rate constants, respectively, and $(BMV)_{e/d}$ represents drug which has reached the viable epidermis and dermis where the steroid elicits its vasoconstriction response [13,14].

The elimination rate constant (k_e) may be determined by the decay of BMV levels in the SC post-removal of the formulation at 6 hours ($A_{6\text{-}30}$):

$$A_{6-30} = A_6 \cdot e^{-k_e \cdot (t-6)} \qquad \text{Eq. 3}$$

where A_6 is the amount of drug in the SC at 6 hours. With this kinetic constant, the zero-order input rate (k_0) can then be deduced from the uptake portion of the DPK profile:

$$A_{0-6} = \frac{k_0}{k_e} \cdot (1 - e^{-k_e \cdot t}) \qquad \text{Eq. 4}$$

2.6. Statistics

Paired two-tailed Student's t-tests were used. P-values < 0.05 were considered statistically significant.

3. Results

3.1. DPK profiles

Figure 3 compares the DPK profiles of BMV delivered from MCT and ME. Significant differences in the amount of drug in the SC were observed at all time points between the two vehicles. ME was clearly the more effective formulation. At 6 hours, SC uptake of BMV from the ME was about 6 µg/cm², compared to only 1 µg/cm² from the MCT formulation. The corresponding BA parameters AUC and A_{max} (Table 2) reflect this observation, and both differ significantly between the two vehicles ($P < 0.05$). The T_{max} values for the two vehicles were not significantly different, on the other hand. The EF_{DPK} (Eq. 1) was 5.05 ± 1.25, clearly indicating that drug delivery into the SC from the ME was superior to that from MCT.

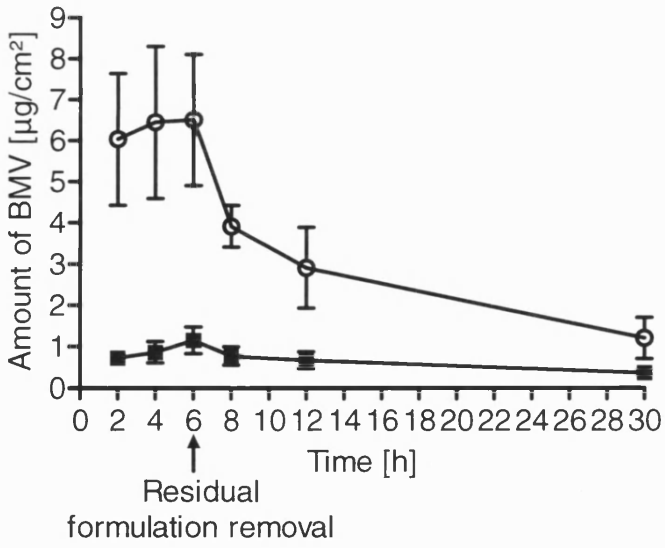


Figure 3: DPK profiles (amount of BMV versus time) of reference (MCT ■) and test (ME ○) formulations. Mean ± SD, n = 6. For the uptake phase, formulations were removed at 2, 4 and 6 hours. For the elimination phase, formulations were maintained to the skin for 6 hours, subsequently removed, and the SC was stripped after a further 2, 6 and 24 hours.

When the DPK profiles were fitted to Eqs. 3 and 4, no difference in the k_e values for the two formulations were found; in contrast, the estimated zero-order input rate from the ME was significantly higher than that from the MCT formulation (Table 2). The vehicle had a clear influence, therefore, on drug uptake.

Table 2: Pharmacokinetic parameters (AUC, A_{max}, T_{max}) and rate constants calculated from the DPK profiles of BMV applied in MCT and ME. Mean ± SD, n = 6.

	AUC(2-30) [µg·h/cm²]	**A_{max}** [µg/cm²]	**T_{max}** [h]	**k_0** [µg/cm²/h]	**k_e** [h^{-1}]
MCT	17.6 ± 3.6	1.15 ± 0.32	5.7 ± 0.8	0.24 ± 0.07	0.04 ± 0.02
ME	86.5 ± 15.1*	7.92 ± 1.50*	4.3 ± 2.0	1.63 ± 0.23*	0.06 ± 0.02

*Significantly different from the reference vehicle, $P < 0.05$

3.2. Blanching profiles

The skin blanching response profiles following 2-hour and 6-hour dose durations are shown in Figure 4. The derived BA parameters are in Table 3. Interestingly, no significant differences between the formulations were observed, in contrast to the DPK results. The longer dose duration generally resulted in greater initial vasoconstriction, more negative values of Δa_{max} and AARC but these differences were not dramatic, and $EF_{VA} \sim 1$. At all experiments, the time at which the maximum response occurred (T_{max}) was 6 hours post-removal of the formulation. This suggests that BMV taken up into the SC during the application period was still transporting through the barrier to the receptors after the vehicle had been removed from the skin. As the DPK experiments clearly show that more BMV is delivered from the ME, relative to MCT, the chromameter results appear to imply that the blanching response has been saturated and cannot be used, in this case, to distinguish the formulations. It is possible that a shorter dose duration might allow some differentiation to be detected.

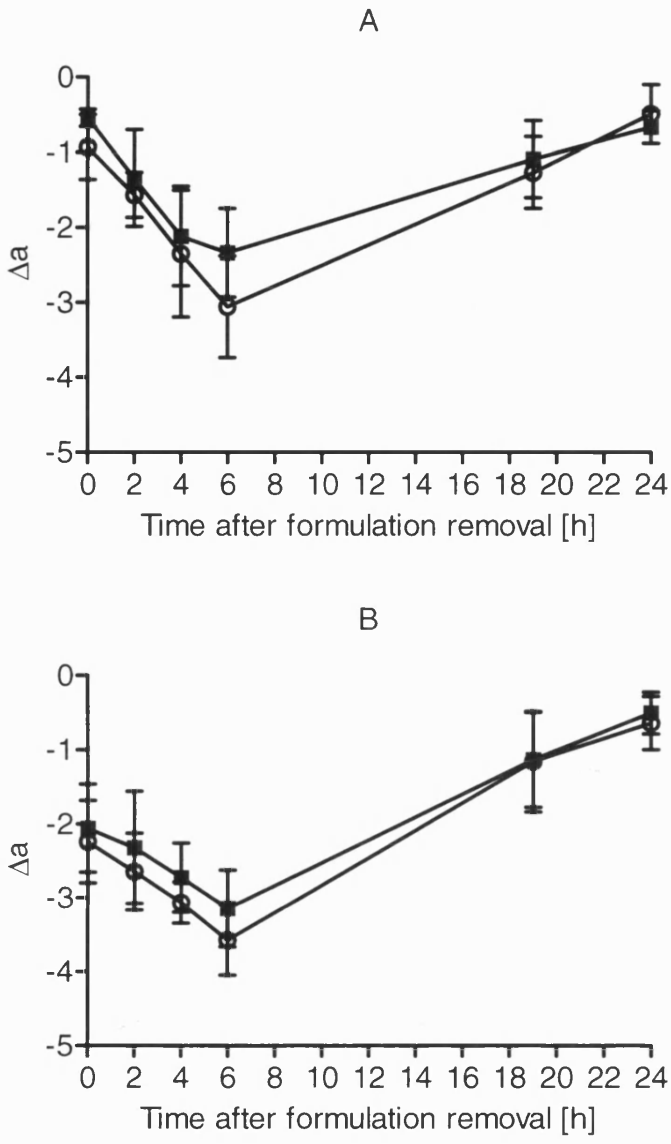


Figure 4: Chromameter (Δa) blanching versus time profiles of MCT (■) and ME (O) after different dose durations: 2 hours (A) and 6 hours (B). Time zero equals time of formulation removal. Mean ± SD, n = 6.

Table 3: Pharmacokinetic parameters (AARC, Δa_{max}, T_{max}) calculated from the blanching profiles of BMV applied in MCT and ME for different dose durations. Mean ± SD, n = 6.

	Dose duration [h]	**AARC(0-24)**	**Δa_{max}**	**T_{max} [h]**
MCT	2	-36.5 ± 8.4	-2.43 ± 0.65	5.3 ± 1.0
	6	-47.2 ± 10.3	-3.15 ± 0.52	6.0 ± 0.0
ME	2	-44.3 ± 9.2	-3.08 ± 0.71	5.7 ± 0.8
	6	-52.5 ± 10.5	-3.57 ± 0.48	6.0 ± 0.0

4. Discussion

The DPK results show that the ME formulation obviously enhances steroid delivery into the SC. The use of microemulsions to improve topical BA has been reported on numerous occasions [15-20], and has been related to the ability of these vehicles to improve drug solubilisation in the intercellular lipids of the SC [17,18,21]. This mechanism would be consistent with the observed EF_{DPK} of about 5 despite the fact that the thermodynamic activities of BMV in the ME and MCT formulations were identical.

As alluded to above, the divergent results from the vasoconstrictor assay suggest that the blanching response had been saturated even at the lower level of drug delivery from the MCT vehicle. Of course, with any pharmacodynamic response, this is a potential issue and it is important, therefore, to operate in the linear part of the 'dose-response' curve whenever quantitative conclusions about BA/BE are to be drawn. The saturation of steroid blanching response has been described on more than one occasion in the literature e.g. [22-24], and it is reasonable to conclude that this has again been observed here. Considerable care should be exercised, therefore, in using the vasoconstrictor assay as a means to validate the DPK approach. Although the DPK approach distinguishes clearly between the two formulations, some clear weaknesses in the Draft Guidance have been exposed, and the document was withdrawn in 2002. As well as concerns about reproducibility of the method between laboratories, there are flaws resulting from the attempt to design a procedure which mirrors that used for oral BA assessment. For orally delivered drugs, the duration of the uptake and clearance phase is controlled by dose, absorption rate, distribution and elimination. For topically delivered drugs, according to the DPK study, the duration of the uptake phase is controlled by the time at which the remaining formulation is removed from the skin surface. As a result A_{max} and T_{max} are strongly dependent on the application time. Moreover, unlike the AUC determined from drug levels in the blood, the AUC measured in the DPK method does not characterise the dose, but instead combines the effect of dose and removal time. This means, that the relative contributions of the uptake and elimination phase to the AUC can be altered significantly by

changing the application time before removing the formulation from the skin surface [25]. These points are illustrated by the results of this study.

Nevertheless, the DPK methodology employed was reproducible and sensitive to differences between the vehicles tested. The procedure involved, while not yet optimised, and perhaps more complicated than the vasoconstrictor assay, is nevertheless straightforward and relatively easy to perform. With modification, therefore, and further development, as already described in recent publications [26,27], the DPK approach may offer a more quantitative and objective strategy for topical BA/BE assessment of TG than the 'classic' vasoconstrictor assay.

5. Acknowledgements

We would like to thank Sebapharma GmbH & Co. KG for providing the microemulsion Mikro 100®.

6. References

[1] Guidance for Industry: Topical dermatologic corticosteroids: In vivo bioequivalence, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1995

[2] Guidance for Industry: Topical dermatologic drug product NDAs and ANDAs-in vivo bioavailability, bioequivalence, in vitro release, and associated studies, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1998

[3] V.P. Shah, G.L. Flynn, A. Yacobi, H.I. Maibach, R.L. Williams, et al., Bioequivalence of topical dermatological dosage forms-methods of evaluation of bioequivalence, Pharm. Res. 1998; 15:167-171

[4] D. Caron, C. Queille-Roussel, V.P. Shah, H. Schaefer, Correlation between the drug penetration and the blanching effect of topically applied hydrocortisone creams in human beings, J. Am. Acad. Dermatol. 1990; 23:458-462

[5] L.K. Pershing, L.D. Lambert, V.P. Shah, S.Y. Lam, Variability and correlation of chromameter and tape-stripping methods with the visual skin blanching assay in the quantitative assessment of topical 0.05% betamethasone dipropionate bioavailability in humans, Int. J. Pharm. 1992; 86:201-210

[6] L.K. Pershing, L.D. Lambert, E.D. Wright, V.P. Shah, R.L. Williams, Topical 0.050% betamethasone dipropionate pharmacokinetic and pharmacodynamic dose-response studies in humans, Arch. Dermatol. 1994; 130:740-747

[7] L.K. Pershing, S. Bakhtian, C.E. Poncelet, J.L. Corlett, V.P. Shah, Comparison of skin stripping, in vitro release, and skin blanching response methods to measure dose response and similarity of triamcinolone acetonide cream strengths from two manufactured sources, J. Pharm. Sci. 2002; 91:1312-1323

[8] T.J. Franz. Study #1, Avita Gel 0.025% vs Retin-A Gel 0.025%: Advisory Committee for Pharmaceutical Sciences Meeting, Center for Drug Evaluation and Research (CDER), Food and Drug Administration (FDA), Rockville, MD; November 29, 2001

[9] L.K. Pershing. Bioequivalence assessment of three 0.025% tretinoin gel products: Dermatopharmacokinetic vs. clinical trial methods. Rockville, MD: Advisory Committee for Pharmaceutical Sciences Meeting, Center for Drug Evaluation and Research (CDER), Food and Drug Administration (FDA), Rockville, MD; November 29, 2001

[10] L.K. Pershing, J.L. Nelson, J.L. Corlett, S.P. Shrivastava, D.B. Hare, V.P. Shah, Assessment of dermatopharmacokinetic approach in the bioequivalence determination of topical tretinoin gel products, J. Am. Acad. Dermatol. 2003; 48:740-751

[11] C.S. Leopold, B.C. Lippold, Enhancing effects of lipophilic vehicles on skin penetration of methyl nicotinate in vivo, J. Pharm. Sci. 1995; 84:195-198

[12] Y.N. Kalia, I. Alberti, N. Sekkat, C. Curdy, A. Naik, R.H. Guy, Normalization of stratum corneum barrier function and transepidermal water loss in vivo, Pharm. Res. 2000; 17:1148-1150

[13] M. Ponec, J.A. Kempenaar, E.R. De Kloet, Corticoids and cultured human epidermal keratinocytes: specific intracellular binding and clinical efficacy, J. Invest. Dermatol. 1981; 76:211-214

[14] R. Marks, J.W. Barlow, J.W. Funder, Steroid-induced vasoconstriction: Glucocorticoid antagonist studies, J. Clin. Endocrinol. Metab. 1982; 54:1075-1077

[15] K. Kriwet, C.C. Mueller-Goymann, Diclofenac release from phospholipid drug systems and permeation through excised human stratum corneum, Int. J. Pharm. 1995; 125:231-242

[16] F.P. Bonina, L. Montenegro, N. Scrofani, E. Esposito, R. Cortesi, E. Menegatti, et al., Effects of phospholipid based formulations on in vitro and in vivo percutaneous absorption of methyl nicotinate, J. Controlled Release 1995; 34:53-63

[17] M.B. Delgado-Charro, G. Iglesias-Vilas, J. Blanco-Mendez, M.A. Lopez-Quintela, J.-P. Marty, R.H. Guy, Delivery of a hydrophilic solute through the skin from novel microemulsion systems, Eur. J. Pharm. Biopharm. 1997; 43:37-42

[18] F. Dreher, P. Walde, P. Walther, E. Wehrli, Interaction of a lecithin microemulsion gel with human stratum corneum and its effect on transdermal transport, J. Controlled Release 1997; 45:131-140

[19] M. Trotta, S. Morel, M.R. Gasco, Effect of oil phase composition on the skin permeation of felodipine from o/w microemulsions, Pharmazie 1997; 52:50-53

[20] M. Kreilgaard, E.J. Pedersen, J.W. Jaroszewski, NMR characterisation and transdermal drug delivery potential of microemulsion systems, J. Controlled Release 2000; 69:421-433

[21] U. Schmalfuß, R. Neubert, W. Wohlrab, Modification of Drug Penetration into Human Skin Using Microemulsions, J. Controlled Release 1997; 46:279-285

[22] R.B. Stoughton, Are generic formulations equivalent to trade name topical glucocorticoids?, Arch. Dermatol. 1987; 123:1312-1314

[23] R.B. Stoughton, K. Wullich, The same glucocorticoid in brand-name products, Arch. Dermatol. 1989; 125:1509-1511

[24] V.P. Shah, C.C. Peck, J.P. Skelly, "Vasoconstriction"-Skin Blanching-Assay for glucocorticoids-a critique, Arch. Dermatol. 1989; 125:1558-1561

[25] B. N'Dri-Stempfer, R.H. Guy, W.C. Navidi, A.L. Bunge. Dermatopharmacokinetic assessment of bioequivalence of topical 0.025% tretinoin gel products: Data re-analysis. In: Perspectives in Percutaneous Penetration; 2006; La Grande Motte; 2006; p. 26

[26] C. Herkenne, A. Naik, Y.N. Kalia, J. Hadgraft, R.H. Guy, Ibuprofen transport into and through skin from topical formulations: In vitro-in vivo comparison, J. Invest. Dermatol. 2006

[27] C. Herkenne, A. Naik, Y.N. Kalia, J. Hadgraft, R.H. Guy, Pig ear skin ex vivo as a model for in vivo dermatopharmacokinetic studies in man, Pharm. Res. 2006; 23:1850-1856

CHAPTER 3

Pharmacodynamics and dermatopharmacokinetics of betamethasone 17-valerate: Assessment of topical bioavailability

Pharmacodynamics and dermatopharmacokinetics of betamethasone 17-valerate: Assessment of topical bioavailability

Sandra Wiedersberg[1], Claudia S. Leopold[2], Richard H. Guy[1]

[1]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK.

[2]Institute for Pharmacy, Department of Pharmaceutical Technology, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany.

Research paper to be submitted

Abstract

Objective: To compare the *in vivo* bioavailability of different formulations of betamethasone 17-valerate (BMV) using the vasoconstrictor assay and the dermatopharmacokinetic (DPK) method.

Methods: BMV was formulated in different vehicles and the drug concentration was adjusted to (i) equal thermodynamic activity and (ii) a range of different concentrations. Skin blanching response was quantified after removal of the formulation over 24 hours with a chromameter. Drug uptake into the stratum corneum (SC) was assessed by tape-stripping.

Results: Same thermodynamic activity leads to similar skin blanching response of BMV delivered from different vehicles, while the DPK profiles distinguish clearly between the formulations. Applying different BMV concentrations resulted in a clear concentration dependence of both the skin blanching response and the drug uptake into the SC. However, the saturable nature of the pharmacodynamic response is obvious.

Conclusion: The vasoconstrictor assay is a well accepted and extensively employed method to determine bioavailability/bioequivalence of TG. Its results have to be analysed with care due to the saturable nature of the pharmacodynamic response. The tape-stripping method, on the other hand, showed reasonable reproducibility and distinguished clearly between

different formulations and different concentrations but more validation work need to be done to establish this method for bioavailability/bioequivalence testing.

1. Introduction

Topical glucocorticoids (TG) are widely used in the treatment of inflammatory skin diseases, such as psoriasis and atopic dermatitis, due to their vasoconstrictive, anti-inflammatory, immunosuppressive and antiproliferative effects. The clinical effectiveness depends on their bioavailability (BA) within the skin at the site of action. For TG, the site of action is within the viable epidermis and dermis, where the glucocorticoid receptors are located [1,2].

Generally, BA of TG is very poor. In part, this is due to inefficient formulations. As well, the absence of quantitative methods with which to assess the rate and extent of drug delivery to the target into the skin is a contributory factor. For the moment, the determination of topical BA is inferred from a demonstration of safety and efficacy via clinical trials.

For TG, bioequivalence (BE) can be estimated either by comparative clinical trials or via application of the vasoconstrictor assay. This latter, non-invasive method is based on the ability of TG to cause measurable skin blanching. Based on this pharmacodynamic response, the U.S. Food and Drug Administration (FDA) published a Guidance document in 1995, entitled "Topical Dermatologic Corticosteroids: *In vivo* Bioequivalence" [3] for BE testing of TG. This Guidance consists of pilot and pivotal studies. The former is carried out to explore the dose duration-response relationship of a reference listed drug (RLD) and to determine the appropriate dose duration for use in the pivotal study. The latter then compares the pharmacodynamic response of the test product with that of the RLD so as to determine whether bioequivalence has, or has not, been achieved.

However, the vasoconstrictor assay is obviously not generally applicable to all topical drugs, and alternative procedures for topical BE assessment are required which are less expensive, less time-consuming and more sensitive than clinical trials. One interesting and increasingly investigated method involves tape-stripping the skin's outermost layer, the stratum corneum (SC). In 1998, the FDA released a Draft Guidance proposing a dermatopharmacokinetic (DPK) method, using tape-stripping,

which evaluates topically applied drug levels in the SC as a function of time post-application and post-removal of the formulation [4,5]. The SC is collected by successive application and removal of adhesive tape and the total amount of drug is subsequently analysed therein. The DPK method assumes that SC drug levels are directly related to those in the viable epidermis and/or dermis, as the SC is typically the rate-determining barrier to percutaneous absorption. In 2002, however, this Draft Guidance was withdrawn mainly because of doubts regarding reproducibility, flaws resulting from the similar design of the approach to oral BE assessment, and criticism that quantification of the amount of SC removed should be better controlled.

As a consequence, a critical re-evaluation of the DPK method is in progress, with a clear objective being to validate the approach. Important progress has been made with respect to quantification and standardization of the amount of SC removed during tape-stripping such that drug concentration profiles across the membrane can now be expressed on the same scale: that is, as a function of the relative position within the SC [6]. Equally, dermatopharmacokinetic parameters, characterizing drug partitioning and diffusivity into and through the SC, can be deduced and used to quantify, respectively, the extent and rate of drug delivery. These advances have been illustrated for terbinafine [7-9] and for ibuprofen [10,11] delivered from different vehicles.

The goal of this study was to explore the challenge of validating the DPK methodology by comparing the assessment of the topical BA of betamethasone 17-valerate using the vasoconstrictor assay and the tape-stripping approach. The sensitivity of the techniques, and their ability to discriminate between different formulations, was examined as a function of the applied drug concentration (and its thermodynamic activity).

2. Materials and methods

2.1. Determination of the saturation level

The saturation level ($C_{s,V}$) of betamethasone 17-valerate (BMV) (Crystal Pharma, Boecillo, Spain) in each vehicle was determined by stirring a suspension of the drug in the formulation at 32 °C until equilibrium was attained (about 72 hours). The samples were centrifuged, diluted either with acetonitrile alone or with acetonitrile/water 60:40 (v/v) and analysed by liquid chromatography (see method below). The saturation level of BMV in light mineral oil was determined using the iterative method of visual clouding. The experiments were performed in triplicate.

2.2. Preparation of the formulations

BMV was dissolved in (i) the reference vehicle, medium chain triglycerides (MCT) (Mygliol 812 N, Synopharm, Barsbüttel, Germany), and in (ii) light mineral oil (LMO) (Synopharm), (iii) the microemulsion Mikro 100® (ME) (Sebapharma, Boppard, Germany), and (iv) Transcutol® P (TCL) (Gattefossé, Saint Priest, France), as test vehicles. Either 15 % (w/w) polypropylene or 10 % (w/w) Aerosil 200® (Sigma-Aldrich, Steinheim, Germany) was used as gelling agent.

In the first part of the study, the BMV concentration was adjusted to 80 % of the saturation level in each vehicle to provide the drug at equivalent thermodynamic activity (Table 1).

Table 1: Saturation level of BMV in different vehicles at 32 °C.
Mean ± SD, n = 3.

Vehicle	Saturation level ($C_{s,V}$) [mg/ml]
LMO	0.0021 ± 0.0003
MCT	2.1 ± 0.2
ME	11.7 ± 0.4
TCL	126.0 ± 1.1

In the second component of the investigation, only the MCT and ME vehicles were evaluated and the BMV concentration was adjusted to different degrees of its saturation level (Table 2).

Table 2: Thermodynamic activities of BMV, expressed as degree of $C_{s,V}$, and concentrations studied in the second set of experiments using ME and MCT formulations only.

Degree of $C_{s,V}$	**0.80**	**0.10**	**0.05**	**0.026**	**0.013**	**0.0064**	**0.0032**
C_V ME [mg/ml]	9.3	1.2	0.6	0.3	0.15	0.07	0.04
C_V MCT [mg/ml]	1.7	0.2			0.027	0.013	

MCT was chosen as the reference vehicle as it was not expected to have any effect on skin barrier function *per se* [12]. BMV was selected as a typical, and frequently used, class 2 TG [13]. BMV has a relatively high molecular weight (476.6 Da) and is quite lipophilic (log(octanol/water partition coefficient) = 3.78).

2.3. Vasoconstrictor assay

Twelve healthy volunteers, aged 21 - 55 years, from whom informed consent was obtained, participated in the study, which was approved by the Ethics Committee of the University of Leipzig. The study followed the "staggered application with synchronized removal" method of the FDA Guidance "Topical Dermatologic Glucocorticoids – *In vivo* Bioequivalence" [3]. 250 µl of each formulation were applied in a 1.2 cm diameter Hill Top Chamber® (Hill Top Research, Cincinnati, OH, USA) which was affixed with adhesive tape. The drug application sites were on the volar forearm, at least 4 cm from the wrist and 4 cm from the antecubital fossa. The amounts of the formulations applied ensured infinite dose conditions.

In the first part of the study, LMO, ME and TCL were compared with the reference vehicle, MCT. The formulations, as well the drug-free vehicles as control, were applied to the skin for dose durations of 2, 3, 4, 5 and 6 hours. The chambers were then removed and excess formulation was cleaned off with a dry paper towel. The skin blanching response was

assessed with a chromameter (CR-300, Minolta, Ahrensburg, Germany) using the a-scale values at various times up to 24 hours following formulation removal. The chromameter was calibrated using an optically white plate immediately before use. Baseline readings were taken at all sites prior to the application of the formulations. The a-scale readings for each drug application site were adjusted for the baseline value and the control and expressed as the change in this parameter (Δa). As the a-scale values decrease with increasing skin blanching, Δa is negative. The degree of response was therefore expressed as the (positive) area above the response curve (AARC) using the trapezoidal rule.

In the second series of experiments, BMV, at different degrees of saturation in MCT and ME, were applied to the forearm for 4 hours (Table 2). After removal of the formulations, the skin blanching response was assessed as before over the 24 hour period after formulation removal.

2.4. Tape-stripping

Six healthy Caucasian volunteers (4 female, 2 male, 23 - 41 years) with no history of dermatological disease participated in these measurements, which were approved by the Salisbury Research Ethics Committee. Written consent was obtained from all subjects.

A single infinite dose volume of 600 µl of each BMV formulation, was applied using a 1.8 cm diameter Hill Top Chamber® on the volar aspect of the forearm, no closer than 4 cm from the bend of the elbow and from the wrist. After a 2-hour application (dose duration), the chambers were removed and excess formulation was cleaned off with a dry paper towel. Immediately after cleaning, the SC at the treated site was progressively removed by repeated adhesive tape-stripping (Scotch Book Tape, 3M, St. Paul, MN, USA). A piece of polypropylene foil with a predefined hole was placed onto the cleaned, treated skin site and affixed with self-adhesive tape. This template ensured that all tape-stripping procedures took place at the same site. The tape (2.5 x 2.5 cm) was applied over this template, using a constant pressure (140 g/cm^2) via a weighted roller and then removed. Up to 20 strips were taken from each site, but the SC was never completely removed. To ascertain the

remaining skin barrier function, transepidermal water loss (TEWL) measurements were performed (AquaFlux V4.7, Biox Systems Ltd., London, UK) during the stripping procedure, which was stopped when TEWL reached 60 g/m²h. Each tape was carefully weighed before and after stripping on a 10-µg precision balance (Mettler AT 261, Greifensee, Switzerland) to determine the mass and thickness of the SC layer removed [14]. BMV in the tape-strips was subsequently extracted quantitatively and analysed by liquid chromatography (see method below). The amount of BMV on each strip was then converted to a concentration corresponding to a specific depth into the SC.

To calculate the total thickness of the SC, the same tape-stripping procedure was performed at an adjacent, untreated skin area with measurements of TEWL after each tape-strip [14]. The amount of SC removed on each tape was again determined gravimetrically. From this mass, the known stripped area, and the density of the tissue (about 1g/cm³ [15]), it was possible to calculate the total thickness of the SC from the x-intercept of a graph of 1/TEWL versus the cumulative thickness of SC removed [14]. Knowing the SC thickness of each subject made it possible to express all BMV concentration profiles as a common function of the relative position (depth) into the SC, greatly facilitating objective comparison [6,16].

2.5. Extraction and HPLC analysis of BMV

Each tape was completely extracted by overnight shaking with 1.0 ml of 60:40 v/v acetonitrile:water (Sigma-Aldrich, Steinheim, Germany). Validation of the extraction procedure involved spiking tape-stripped samples of untreated SC with a known quantity of BMV. Recovery was 96.9 ± 3.4 % (n = 5). BMV in the various samples was quantified by high-performance liquid chromatography (HPLC) analysis (Dionex, Munich, Germany) using a Lichrospher® 100 RP-18 (4 x 125 mm) column (Hichrom, Reading, UK) with UV detection at 240 nm. The mobile phase was degassed acetonitrile:deionised water (60:40 v/v) and was delivered at a flow rate of 1 ml/min in a 50-µl sample loop. The retention time of BMV at 25 °C was

~ 3.8 minutes. A calibration curve was generated with the pure compound at 5 different concentrations. The detection limit was 0.03 µg/ml.

2.6. Analysis of the concentration profile data

The SC distribution profiles of BMV (i.e. drug concentration (C_x) as a function of position (x) within the SC and time (t)) were fitted to the appropriate solution of Fick's second law of diffusion [17]:

$$C_x = KC_V\left[1-\frac{x}{L}-\frac{2}{\pi}\sum_{n=1}^{\infty}\frac{1}{n}\sin(n\pi\frac{x}{L})\exp(-\frac{D}{L^2}n^2\pi^2t)\right] \qquad \text{Eq. 1}$$

where C_V is the BMV concentration in the vehicle, K is the apparent partition coefficient of BMV between the SC and the applied vehicle, and D is the diffusivity of the drug in the SC of total thickness L.

The analysis assumes the following boundary conditions: (i) at the skin surface (x = 0), for the entire duration of the experiment, the BMV concentration is $K \cdot C_V$; (ii) at t = 0, the SC contains no drug; and (iii) at the inner surface of the SC (x = L), perfect 'sink' conditions exist for the drug.

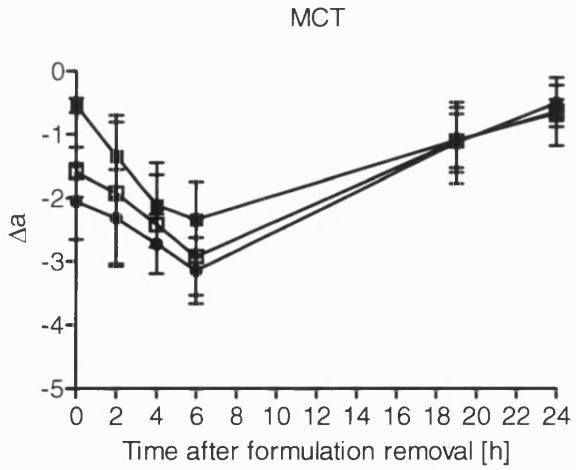
Fitting the experimental data to Eq. 1 (using GraphPad Prism® 4.03 software, San Diego, CA, USA) allowed estimates of K and D/L^2 to be derived. The latter has units of a first-order rate constant ($time^{-1}$), and is a 'classic' diffusion parameter derived from these experiments [18]. Integration of Eq. 1 yields the area under the drug concentration profile (AUC):

$$AUC = \int_0^1 C_x d\left(\frac{x}{L}\right) = KC_V\left[\frac{1}{2}-\frac{4}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left(-\frac{D}{L^2}(2n+1)^2\pi^2t\right)\right] \qquad \text{Eq. 2}$$

3. Results and discussion

3.1. Influence of vehicle and dose duration on skin blanching

Selected mean blanching response versus time profiles, as a function of dose duration and vehicle, are shown in Figure 1. BMV was applied at the same thermodynamic activity (80 % of saturation) in each formulation. The chromameter readings were baseline-adjusted and untreated control site corrected and are expressed as Δa values. The blanching response increased with time for all formulations post-removal reaching a maximum after 4 - 6 hours. It would appear, therefore, that drug taken up into the SC during the application period was still being released to the site of vasoconstrictive response post-removal of the delivery system, a manifestation of the phenomenon often referred to, in the literature, as a reservoir effect [19-22].



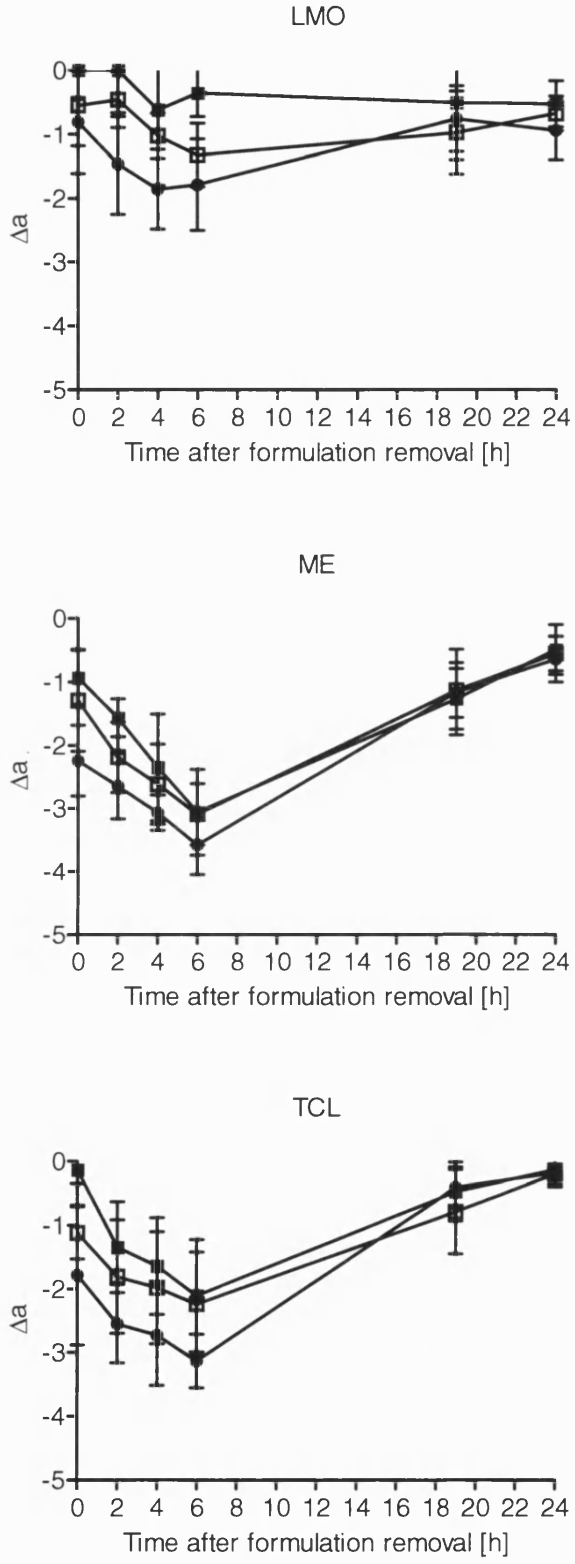


Figure 1: Selected blanching response (Δa) versus time profiles provoked by BMV delivered in MCT, LMO, ME and TCL following 2-h ■, 4-h □ and 6-h ● dose durations. Mean ± SD, n = 6.

The skin blanching response increased with increasing dose duration. Analysis of variance indicated that both the vehicle and the dose duration had a significant influence on the AARC values ($P < 0.05$, two-way ANOVA). Figure 2 summarizes these results and shows clearly that the drug delivered from LMO shows the lowest pharmacodynamic response at all investigated dose durations. On the other hand, the blanching response profiles induced by BMV delivered from MCT, ME and TCL were similar, and not significantly different from each other. While these latter findings would be consistent with the anticipated equivalence of delivery from vehicles containing the drug at similar thermodynamic activities, the poorer performance of LMO, which was not seen in earlier work [23], suggests that this formulation is retarding BMV transport in some way. However, because these conclusions are based on measurements of a pharmacodynamic response, which is known to be saturable (as is clearly seen from Figure 2), an independent approach to evaluate drug delivery is desirable and comprises the justification for the tape-stripping component of this work described below.

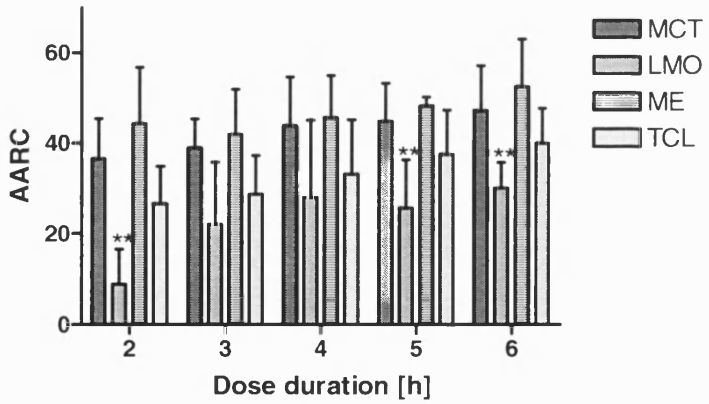


Figure 2: Derived AARC values as a function of dose duration and vehicle. Mean ± SD, n = 6. Significant differences ($P < 0.01$) relative to the reference formulation (MCT) are shown by the double asterisks.

3.2. Influence of vehicle on drug uptake into SC

Uptake of BMV from the different vehicles into the SC, determined by tape-stripping, yielded the results collected in Figure 3 for MCT (the reference formulation), ME and TCL. Data from LMO could not be obtained because the levels of drug extracted from the tape-strips were not quantifiable. Fitting Eq. 1 to the profiles generated individual values of K and D/L^2, the means (± SD) of which are in Table 3. AUC values were calculated from these parameters using Eq. 2 and are also included in Table 3. BMV delivery was significantly different between all formulations with TCL > ME > MCT (ANOVA, $p < 0.0001$). In terms of AUC, TCL out-performed MCT by a factor of more than 60-fold, while ME achieved a 5-fold improvement relative to the reference formulation. Interestingly, the values of K and D/L^2 deduced from the concentration profiles were not significantly different between MCT, ME and TCL (Table 3). In contrast, the deduced saturation concentrations of BMV in the SC ($C_{s,SC}$) were highly vehicle-dependent implying that components of the ME and TCL formulations had also been taken up into the SC in sufficient quantities to alter the drug's solubility in the barrier. Such behaviour has been previously reported for Transcutol® [24-26] and for other cosolvents, such as propylene glycol [11,27].

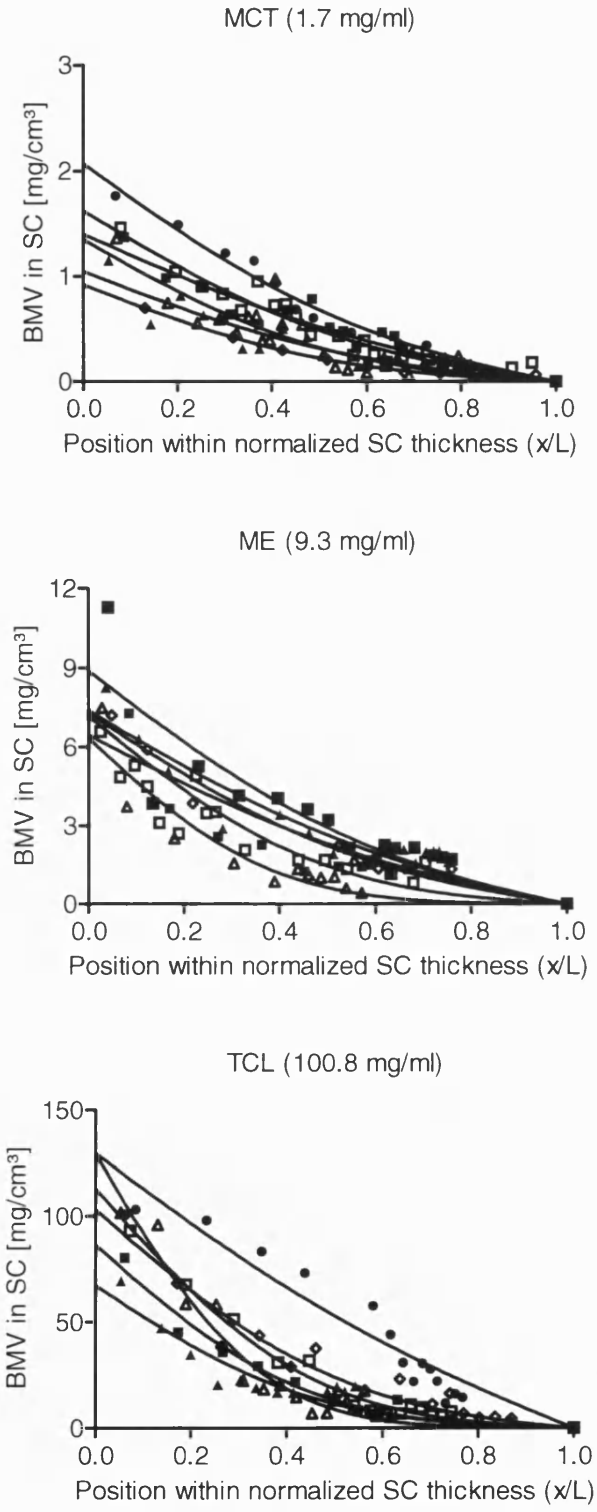


Figure 3: BMV concentration profiles across the SC following a 2-hour application of the drug at 80 % of saturation in three different vehicles: MCT, ME and TCL. The individual profiles from 6 volunteers are shown, together with the best fits to the data of Eq. 1.

Table 3: Tape-stripping experimental results (mean ± SD, n = 6) for BMV delivered from 4 vehicles following a 2-hour application. The drug concentration in each formulation equalled 80 % of its saturation level.

Vehicle	C_V [mg/ml]	K[a]	D/L^2 [h^{-1}][a]	$C_{s,SC}$ [mg/ml][b]	AUC [mg/cm^3][c]
MCT	1.7	0.83 ± 0.24	0.058 ± 0.013	1.68 ± 0.49*	0.51 ± 0.18*
ME	9.3	0.78 ± 0.10	0.056 ± 0.021	9.13 ± 1.15*	2.61 ± 0.67*
TCL	100.8	1.04 ± 0.24	0.042 ± 0.028	131.07 ± 30.78*	32.51 ± 13.25*
LMO	0.0017	n.d.[d]	n.d.[d]	-	n.d.[d]

[a]Obtained by fitting experimental data to Eq. 1.

[b]$C_{s,SC} = K \cdot C_{s,V}$.

[c]From Eq. 2 using the corresponding value of K and D/L^2.

[d]Not determined as BMV could not be quantified in tape-strip extracts.

*Significantly different from each other ($p < 0.0001$).

3.3. Influence of drug concentration applied on skin blanching

The first series of vasoconstrictor experiments, taken together with the tape-stripping results, strongly suggested that the pharmacodynamic response was saturated when BMV was applied at 80 % of its maximum thermodynamic activity. Therefore, a second set of experiments was performed, using the MCT and ME formulations, to examine skin blanching when the drug was administered at a lower dose. A single dose duration of 4 hours was chosen and the vasoconstriction was followed over the next 24 hours. The results show a clear concentration dependence of the response, as has been previously suggested [28]. Figure 4 expresses the data in terms of the AARC as a function of either the BMV concentration in the vehicle or its degree of saturation.

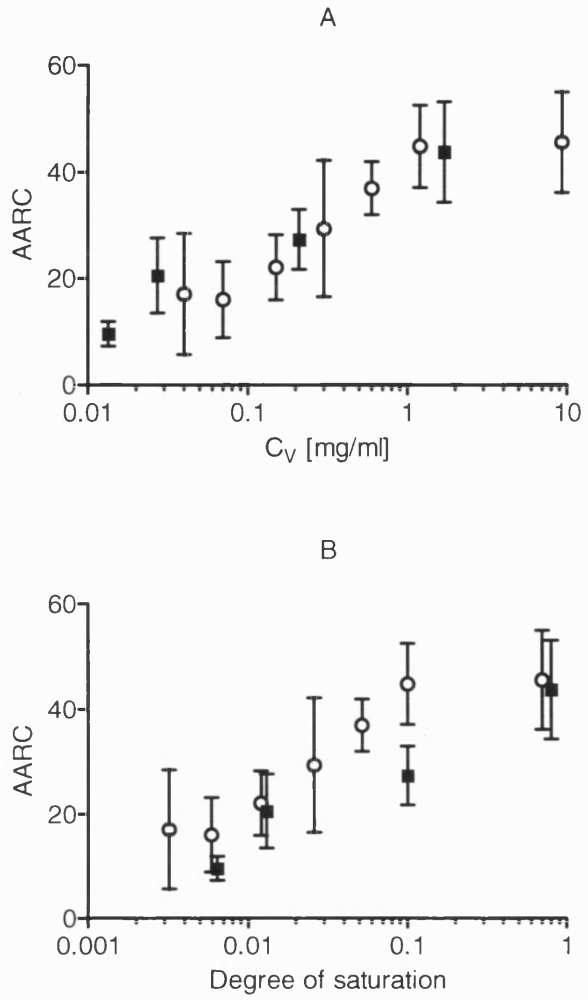


Figure 4: Skin blanching response (AARC) as a function of (A) BMV concentration C_V, and (B) degree of saturation in the vehicles studied (MCT (■) and ME (○)) following a 4-hour application. Mean ± SD, n = 6.

3.4. Influence of drug concentration applied on uptake into the SC

It was then logical to determine whether BMV delivery into the SC showed a concentration dependence and to determine the nature of the relationship. Again, using the MCT and ME vehicles, the SC uptake of drug was evaluated as a function of concentration in the formulation. The results, following a 2-hour application are summarized in Figure 5 (and Figure 3) and Table 4. The lowest concentrations considered were those that resulted in quantifiable amounts of drug in the tape-strips; for this reason, only one other MCT formulation was studies while two others mere possible for ME.

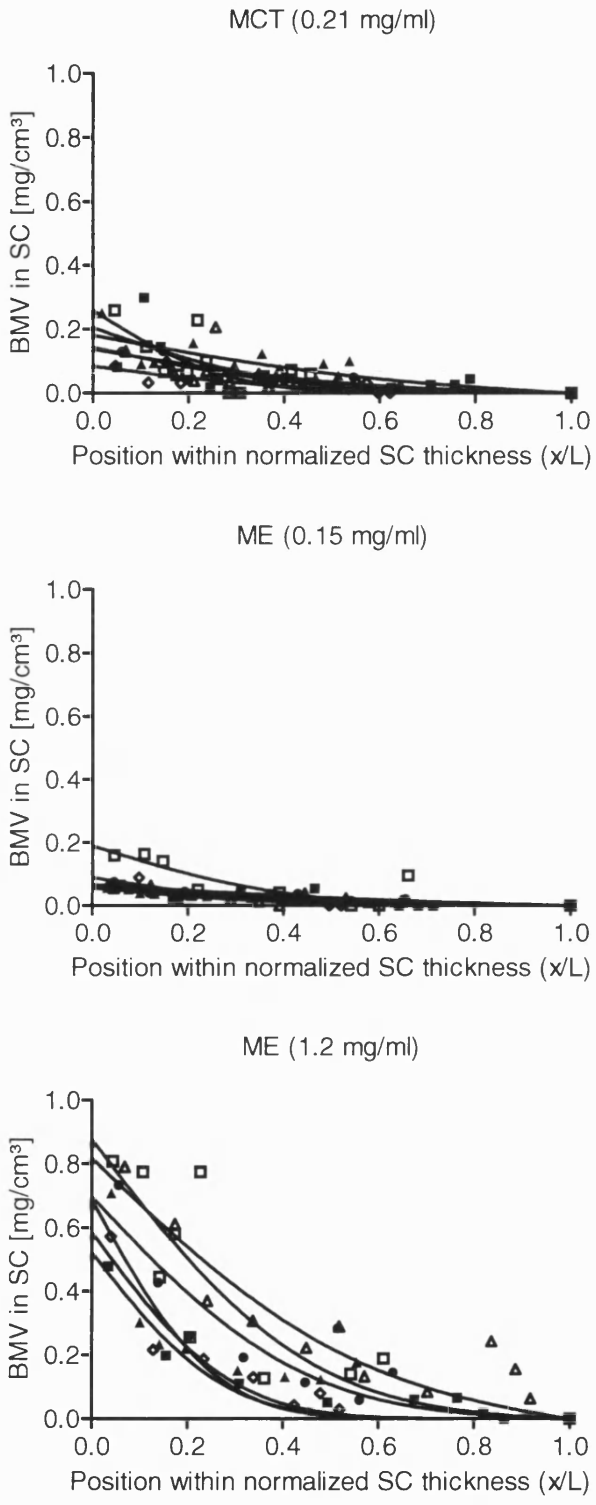


Figure 5: BMV concentration profiles across the SC following a 2-hour application of the drug in MCT and ME vehicles at different concentrations. The individual profiles from 6 volunteers are shown, together with the best fits of the data to Eq. 1

It is first noted that the estimated values of K, D/L^2 were not significantly different from those derived from the results for the vehicles which contained BMV at 80 % of its saturation level (Table 4). In contrast, the AUC values were decreased when the drug was applied at lower concentrations, with the reductions observed being more or less proportional to the corresponding change in BMV thermodynamic activity. For example, an 8-fold decrease in BMV level in the MCT vehicle resulted in an approximately order of magnitude reduction in AUC; similarly, when the drug concentration in ME was lowered by ~ 60-fold, the AUC decreased by about a factor of 80. It follows that, unlike the skin blanching response, drug uptake into the SC from a vehicle, in which the compound is below its solubility, is not saturable.

Table 4: Tape-stripping experimental results (mean ± SD, n = 6) for BMV delivered from MCT and ME vehicles following a 2-hour application. The drug concentrations in each formulation were modified to significantly alter the BMV thermodynamic activity.

Vehicle	C_V [mg/ml]	Degree of $C_{s,V}$	K[a]	D/L^2 $[h^{-1}]$[a]	$C_{s,SC}$ [mg/ml][b]	AUC[c] [mg/cm³]
MCT	1.7	0.80	0.83±0.24	0.058±0.013	1.68±0.49	0.51±0.18
	0.21	0.10	0.80±0.29	0.036±0.020	1.69±0.61	0.05±0.02*
ME	9.3	0.80	0.78±0.10	0.056±0.021	9.13±1.15	2.61± 0.67
	1.2	0.10	0.58±0.11	0.025±0.016	6.81±1.30	0.17±0.08*
	0.15	0.013	0.59±0.33	0.042±0.024	6.95±3.96	0.03±0.01*

[a]Obtained by fitting experimental data to Eq. 1.

[b]$C_{s,SC} = K \cdot C_{s,V}$.

[c]From Eq. 2 using the corresponding values of K and D/L^2.

*Significantly different ($P < 0.05$) from the corresponding value at $0.80 \cdot C_{s,V}$.

This conclusion is substantiated when the skin blanching AARC is plotted against the SC uptake AUC determined by tape-stripping (Figure 6). When all relevant data are included (i.e., one concentration for TCL, two for MCT and three for ME), it is clear that the delivery of BMV into the SC is affected by both the thermodynamic activity of the drug and by specific

vehicle-skin interactions. No evidence for saturation of the barrier can be identified. In contrast, despite a nearly three order change in AUC, the vasoconstriction response changed by less than a factor of 10 and was clearly saturable.

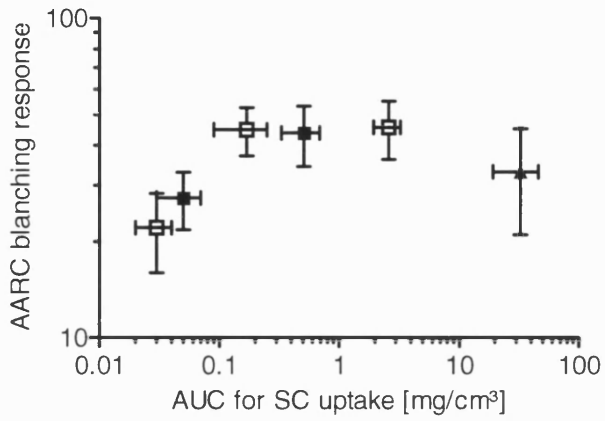


Figure 6: Skin blanching AARC (determined after a 4-hour dose duration) plotted against the corresponding AUC for SC uptake determined by tape-stripping after a 2-hour application. Data for BMV delivered from MCT (closed squares), ME (open squares) and TCL (closed triangles) are shown. Mean ± SD, n = 6.

4. Conclusions

This work demonstrates that the evaluation of topical BA is complex and may be sensitive to the methodology employed. While the vasoconstrictor assay has been employed extensively for corticosteroids, the saturable nature of the response means that results from such experiments must be analysed with care (as indeed they have been in most occasions in the past). The tape-stripping, or DPK approach, would appear to offer a reliable metric with which to quantify transfer of drug from the vehicle to the SC. The critical validation of this measure to clinical outcome remains a long-term objective that will probably be achieved on a case by case (or drug class by drug class) basis. Methodological questions remain here as well; for example, the SC recoveries are dependent upon the surface cleaning procedure at the end of the application period being both efficient and benign (i.e., not encouraging penetration). Further work is essential to explore this question, in particular the potential significance of formulation which may become entrapped in skin 'furrows' and erroneously considered to be absorbed.

5. Acknowledgements

We would like to thank Sebapharma GmbH & Co. KG and Gattefossé for providing the microemulsion Mikro 100® and Transcutol®, respectively.

6. References

[1] M. Ponec, J.A. Kempenaar, E.R. De Kloet, Corticoids and cultured human epidermal keratinocytes: specific intracellular binding and clinical efficacy, J. Invest. Dermatol. 1981, 76:211-214

[2] R. Marks, J.W. Barlow, J.W. Funder, Steroid-induced vasoconstriction: Glucocorticoid antagonist studies, J. Clin. Endocrinol. Metab. 1982, 54:1075-1077

[3] Guidance for Industry: Topical dermatologic corticosteroids: In vivo bioequivalence, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1995

[4] Guidance for Industry: Topical dermatologic drug product NDAs and ANDAs-in vivo bioavailability, bioequivalence, in vitro release, and associated studies, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1998

[5] V.P. Shah, G.L. Flynn, A. Yacobi, H.I. Maibach, R.L. Williams, et al., Bioequivalence of topical dermatological dosage forms-methods of evaluation of bioequivalence, Pharm. Res. 1998, 15:167-171

[6] Y.N. Kalia, I. Alberti, N. Sekkat, C. Curdy, A. Naik, R.H. Guy, Normalization of stratum corneum barrier function and transepidermal water loss in vivo, Pharm. Res. 2000, 17:1148-1150

[7] I. Alberti, Y.N. Kalia, A. Naik, J.-D. Bonny, R.H. Guy, In vivo assessment of enhanced topical delivery of terbinafine to human stratum corneum, J. Controlled Release 2001, 71:319-327

[8] I. Alberti, Y.N. Kalia, A. Naik, J.-D. Bonny, R.H. Guy, Effect of ethanol and isopropyl myristate on the availability of topical terbinafine in human stratum corneum, in vivo, Int. J. Pharm. 2001, 219:11-19

[9] I. Alberti, Y.N. Kalia, A. Naik, R.H. Guy, Assessment and prediction of the cutaneous bioavailability of topical terbinafine, in vivo, in man, Pharm. Res. 2001, 18:1472-1475

[10] C. Herkenne, A. Naik, Y.N. Kalia, J. Hadgraft, R.H. Guy, Ibuprofen transport into and through skin from topical formulations: In vitro-in vivo comparison, J. Invest. Dermatol. 2006

[11] C. Herkenne, A. Naik, Y.N. Kalia, J. Hadgraft, R.H. Guy, Pig ear skin ex vivo as a model for in vivo dermatopharmacokinetic studies in man, Pharm. Res. 2006, 23:1850-1856

[12] C.S. Leopold, B.C. Lippold, Enhancing effects of lipophilic vehicles on skin penetration of methyl nicotinate in vivo, J. Pharm. Sci. 1995, 84:195-198.

[13] British National Formulary. London: British Medical Association and the Royal Pharmaceutical Society of Great Britain, 2004

[14] Y. Kalia, F. Pirot, R. Guy, Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum in vivo, Biophys. J. 1996, 71:2692-2700

[15] R.L. Anderson, J.M. Cassidy, Variations in physical dimensions and chemical composition of human stratum corneum, J. Invest. Dermatol. 1973, 61:30-32

[16] Y.N. Kalia, I. Alberti, A. Naik, R.H. Guy, Assessment of topical bioavailability in vivo: The importance of stratum corneum thickness, Skin Pharmacol. Appl. Skin Physiol. 2001, 14:82-86

[17] J. Crank. Diffusion in a plane sheet. in: J. Crank, (Eds.), The mathematics of diffusion, 2nd ed. London: Oxford University Press, 1975, pp. 44-68

[18] F. Pirot, Y.N. Kalia, A.L. Stinchcomb, G. Keating, A. Bunge, R.H. Guy, Characterization of the permeability barrier of human skin in vivo, PNAS 1997, 94:1562-1567

[19] C.F.H. Vickers, Existence of reservoir in the stratum corneum, Arch. Dermatol. 1963, 88:72-75

[20] R.D. Carr, R.G. Wieland, Corticosteroid reservoir in the stratum corneum, Arch. Dermatol. 1966, 94:81-84

[21] D. Dupuis, A. Rougier, R. Roguet, C. Lotte, G. Kalopissis, In vivo relationship between horny layer reservoir effect and percutaneous absorption in human and rat, J. Invest. Dermatol. 1984, 82:353-356

[22] A. Rougier, D. Dupuis, C. Lotte, R. Roguet, H. Schaefer, In vivo correlation between stratum corneum reservoir function and percutaneous absorption., J. Invest. Dermatol. 1983, 81:275-278

[23] M. Bach, B.C. Lippold, Influence of penetration enhancers on the blanching intensity of betamethasone 17-benzoate, Int. J. Pharm. 1998, 168:97-108

[24] D.A. Godwin, N.-H. Kim, L.A. Felton, Influence of Transcutol(R) CG on the skin accumulation and transdermal permeation of ultraviolet absorbers, Eur. J. Pharm. Biopharm. 2002, 53:23-27

[25] P. Mura, M.T. Faucci, G. Bramanti, P. Corti, Evaluation of transcutol as a clonazepam transdermal permeation enhancer from hydrophilic gel formulations, Eur. J. Pharm. Sci. 2000, 9:365-372

[26] J.E. Harrison, A.C. Watkinson, D.M. Green, J. Hadgraft, K. Brain, The relative effect of Azone and Transcutol on permeant diffusivity and solubility in human stratum corneum, Pharm. Res. 1996, 13:542-546

[27] B. Bendas, U. Schmalfuß, R. Neubert, Influence of propylene glycol as cosolvent on mechanism of drug transport from hydrogels, Int. J. Pharm. 1995, 116:19-30

[28] V.P. Shah, C.C. Peck, J.P. Skelly, "Vasoconstriction"-Skin Blanching-Assay for glucocorticoids-a critique, Arch. Dermatol. 1989, 125:1558-1561

CHAPTER 4

Dermatopharmacokinetics of betamethasone 17-valerate: Influence of formulation viscosity and skin surface cleaning procedure

Dermatopharmacokinetics of betamethasone 17-valerate: Influence of formulation viscosity and skin surface cleaning procedure

Sandra Wiedersberg[1], Claudia S. Leopold[2], Richard H. Guy[1]

[1]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK.

[2]Institute for Pharmacy, Department of Pharmaceutical Technology, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany.

Research paper to be submitted

Abstract

Objective: To compare the *in vivo* distribution profiles of betamethasone 17-valerate (BMV) across the stratum corneum (SC) following (a) delivery from gelled and un-gelled formulations, and (b) two different skin cleaning procedures at the end of the application period.

Methods: BMV was dissolved in gelled and un-gelled vehicles comprising either medium chain triglycerides (MCT) or a brand microemulsion (ME). The BMV concentration was adjusted to 80 % of the saturation level and applied to the ventral forearm of healthy human subjects. After a 2-hour application, the treated skin site was cleaned either with a dry paper towel, or by an isopropyl alcohol swab, and the SC was then progressively removed by repeated adhesive tape-stripping.

Results: The distribution profiles of BMV across the SC showed that drug delivery from the ME was significantly superior to that from MCT. When the skin surface was cleaned by dry-wiping, the areas under the SC concentration versus normalized position within the SC curve (AUCs) of the gelled formulations, respectively, were 2.54 (± 0.69) and 0.53 (± 0.18) mg/cm^3; for the un-gelled formulations, the corresponding AUCs were significantly smaller, but showed the same relative difference (5 to 6-fold) between the two vehicles 0.97 (± 0.42) and 0.17 (± 0.10) mg/cm^3. Removing excess

formulation more aggressively with isopropyl alcohol resulted in AUCs for ME and MCT, respectively, of 0.54 (± 0.16) and 0.20 (± 0.12) mg/cm^3.

Conclusion: Assessment of the SC distribution profile of BMV showed reasonable reproducibility and a clear ability to distinguish between the two formulations. The gelling agent and the method of surface cleaning had a significant influence on the apparent extent of drug delivered and on the deduced, apparent partition coefficient. Excess gelled formulation may be trapped in the skin 'furrows' and requires an efficient skin cleaning procedure to ensure its complete removal.

1. Introduction

The tape-stripping, or dermatopharmacokinetic (DPK), method is attracting increasing attention as a method with which to assess the rate and extent of topical drug bioavailability in the stratum corneum (SC) [1-3]. This approach, first described by the U.S. Food and Drug Administration (FDA), has been proposed for the bioequivalence testing of topically applied drugs [4]. In 2002, however, the Draft Guidance [5] was withdrawn because of doubts regarding the reproducibility between laboratories and, currently, the method is being re-evaluated and improved [6].

The DPK concept involves determination of the amount of drug present in the SC as a function of time post-application and post-removal of the formulation under examination. A key experimental requirement before this evaluation is made is to ensure that the residual formulation on the skin surface is completely removed before tape-stripping is commenced. The cleaning procedure must be efficient, but not so aggressive as to 'drive' material into the barrier nor to extract it therefrom. Clearly, different types of formulation pose different levels of challenge: for example, it might be anticipated that a simple lotion will be easier to clean off than an oleaginous ointment. The SC surface is not flat; it possesses macroscopic furrows, which run parallel to the surface of the skin [7]. It has been argued that the quantified amount of drug in the SC tape-strips will include the portion deposited in 'furrows' as well as that which has penetrated into the SC. The putative excess formulation in the 'furrows' may complicate the interpretation of data obtained after tape-stripping.

The aim of this study, therefore, was to investigate whether the cleaning procedure of the treated skin site before stripping altered the DPK profile of a typical topical drug (betamethasone 17-valerate) applied in two different formulations. Specifically, cleaning the surface with a dry paper towel (as described by the FDA) was compared to wiping with an isopropyl alcohol swab. In addition, the SC concentration profiles of the same drug, resulting from the administration of the same formulations, which were either applied as liquids, or as semi-solids following gelling with an appropriate polymeric excipient, were evaluated.

2. Materials and methods

2.1. Materials

Betamethasone 17-valerate (BMV) (Crystal Pharma, Boecillo, Spain) was dissolved either in the reference vehicle consisting of medium chain triglycerides (MCT) (Mygliol 812 N, Synopharm, Barsbüttel, Germany) or in the microemulsion Mikro 100® (ME) (Sebapharma, Boppard, Germany). When appropriate, MCT and ME were gelled with 15 % (w/w) polypropylene, and 10 % (w/w) Aerosil 200® (Sigma-Aldrich, Steinheim, Germany), respectively. The BMV concentration was adjusted to 80 % of the saturation level $C_{s,V}$ (i.e., to a constant thermodynamic activity), equivalent to 1.7 mg/ml in MCT and 9.3 mg/ml in ME [8].

2.2. Tape-stripping procedure

6 healthy volunteers, 4 female, 2 male (26 - 41 years of age), with no history of skin disease, participated in the study, which was approved by the local research ethics committee (Salisbury Research Ethics Committee). Informed consent was obtained from each subject.

600 µl of the gelled formulations were applied using a 1.8 cm diameter Hill Top Chamber® (Hill Top Research, Cincinnati, OH, USA) on the forearm, at least 4 cm from either the wrist or the bend of the elbow. In a first series of experiments, after an application time of 2 or 6 hours, the SC at the treated skin site was cleaned with a dry paper towel. Then, the SC was progressively removed by repeated adhesive tape-stripping (Scotch Book Tape, 3M, St. Paul, MN, USA). In a second set of studies, the same formulations were applied and the same procedure adopted, except that the skin surface cleaning involved first wiping with a dry paper towel followed by twice wiping a 70 % v/v isopropyl alcohol swab (Sterets swab, Seton Healthcare, Oldham, UK) across the treated skin site. In a third protocol, the MCT and ME formulations were applied as un-gelled liquids. A foam tape (3M, St. Paul, MN, USA), into which a 2.0 cm diameter hole had been cut, acted as a template to constrain the vehicles, and the system was covered by an occlusive tape (Blenderm™, 3M, Neus, Germany) to prevent any loss. After a

2-hour application, the template was removed and excess formulation was cleaned away with a dry paper towel.

Briefly, the tape-stripping procedure involved the following steps. A piece of polypropylene foil, into which a predefined hole had been cut, was placed onto the cleaned, treated skin site and affixed by a piece of self-adhesive tape. This template ensured that all tape-strips were removed from the same site. The tape (2.5 x 2.5 cm) was applied to this template, pressed down with a constant pressure (140 g/cm^2) using a weighted roller and then removed. Up to 20 strips were taken from each treated site, such that the SC was never completely removed. To check skin barrier function, transepidermal water loss (TEWL) measurements were performed (AquaFlux V4.7, Biox Systems Ltd., London, UK) during the stripping procedure, which was stopped if TEWL reached 60 g/m^{2}h. Each tape was carefully weighed before and after stripping on a 10-µg precision balance (Mettler AT 261, Greifensee, Switzerland) to determine the mass and thickness of the SC layer removed [9]. BMV in the tape-strips was subsequently extracted quantitatively and analysed by high-performance liquid chromatography (HPLC) (see method below). The amount of BMV on each strip could then be converted to a concentration within that removed layer of the SC.

To calculate the total thickness of the SC, the same tape-stripping procedure was performed at an adjacent, untreated skin area with periodic measurements of TEWL after each tape-strip [9]. Pre-weighed tapes were used which were re-weighed after a SC layer had been detached to assess the amount of SC removed. From this mass, and knowing the stripping area and the density of the SC [10], it was possible to calculate the total thickness of the SC from the x-axis intercept of a graph of 1/TEWL versus the cumulative thickness of the SC removed. In this way, the drug concentration profile across the SC could be displayed in a consistent fashion for all subjects, as a function of relative position (or depth) into the barrier.

2.3. Extraction and HPLC analysis of BMV

Each tape was placed into a 2 ml tube, and was extracted with 1.0 ml of 60:40 (v/v) acetonitrile/water by shaking overnight. Validation of the

extraction process involved spiking tape-stripped samples of untreated SC with 20 µl of a solution of known drug concentration. Drug recovery was 96.9 ± 3.4 % (n = 5). BMV in the various samples was quantified by HPLC (Dionex, Sunnyvale, CA, USA) with UV detection at 240 nm using a Lichrospher® 100 RP-18 column (4 x 125 mm) (Hichrom, Reading, UK). The mobile phase was degassed acetonitrile/water (60:40 v/v) delivered at a flow rate of 1 ml/min in a 50-µl sample loop. The retention time of BMV at 25 °C was ~ 3.8 minutes. A calibration curve was generated using solutions of the pure compound at 5 different concentrations. The detection limit was 0.03 µg/ml. The amount of BMV recovered from the tape-strips was expressed in terms of amount of drug per unit SC volume (mg/cm³).

2.4. Analysis of the SC distribution profile

The SC concentration (C_x) versus normalized depth (x/L) profiles of BMV were fitted to the appropriate solution of Fick´s second law of diffusion (Eq. 1) [11]:

$$C_x = KC_V\left[1-\frac{x}{L}-\frac{2}{\pi}\sum_{n=1}^{\infty}\frac{1}{n}\sin(n\pi\frac{x}{L})\exp(-\frac{D}{L^2}n^2\pi^2t)\right] \qquad \text{Eq. 1}$$

The boundary conditions necessary are: (i) the SC is free of drug at t = 0, (ii) the drug concentration at the skin surface is constant (infinite dose conditions), and (iii) the viable epidermis at the lower surface of the SC provides perfect sink conditions for the drug. C_V is the BMV concentration in the vehicle. From the best fit of the experimental values to Eq. 1, the SC-vehicle partition coefficient (K) and the diffusivity parameter (D/L^2) across the SC of pathlength L, were obtained. Subsequently, using the derived parameters, K and D/L^2, Eq. 1 was integrated across the SC thickness (i.e., from x/L = 0 to x/L = 1) to yield the area under the concentration profile (AUC).

$$AUC = \int_0^1 C_x d\left(\frac{x}{L}\right) = KC_V\left[\frac{1}{2}-\frac{4}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left(-\frac{D}{L^2}(2n+1)^2\pi^2t\right)\right] \qquad \text{Eq. 2}$$

The derived AUC values were used to compare the relative bioavailability of BMV from the two vehicles and from their quotient (AUC_{ME}/AUC_{MCT}), a penetration enhancement factor (EF) was calculated.

2.5. Statistics

Statistical differences were assessed by two-tailed, Student's t-test and by ANOVA, followed by a Bonferroni's multiple comparison test, using GraphPad Prism 4.01 software (San Diego, CA, USA).

3. Results and discussion

Several factors, such as skin hydration, vehicle composition, cohesion between corneocytes, and inter-individual differences in total SC thickness [12-15], can influence the amount of SC that is removed by a single tape-strip. It follows that this quantity is not linearly proportional to the number of tape-strips removed and that normalization of the SC thickness removed is a prerequisite to facilitate comparison between subjects and between formulations.

The individual SC distribution profiles of BMV after a 2-hour application time of the gelled MCT and ME formulations are in Figure 1. The ME clearly and significantly (two-tailed t-test, $P < 0.05$) increased the extent of drug delivery into the SC, relative to that achieved from the MCT vehicle, as reflected in the AUCs observed: 2.61 (± 0.67) and 0.51 (± 0.18) mg/cm^3 for ME and MCT, respectively, corresponding to an EF of 5.6 ± 2.2.

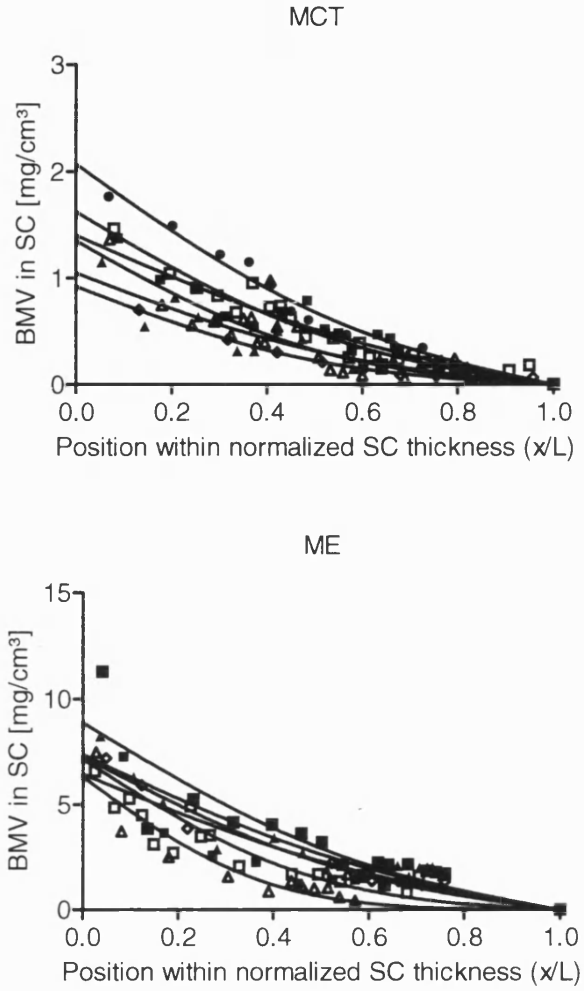


Figure 1: SC distribution profiles of BMV delivered from gelled MCT and ME formulations following a 2-hour application. The skin surface was cleaned by wiping with a dry paper towel. The best fits of Eq. 1 to the individual experimental data points are shown (n = 6).

When the same formulations were administered for a longer period of 6 hours, BMV from both MCT and ME penetrated further into the SC and the profiles became more linear (indicating the approach to steady-state transport conditions) (Figure 2).

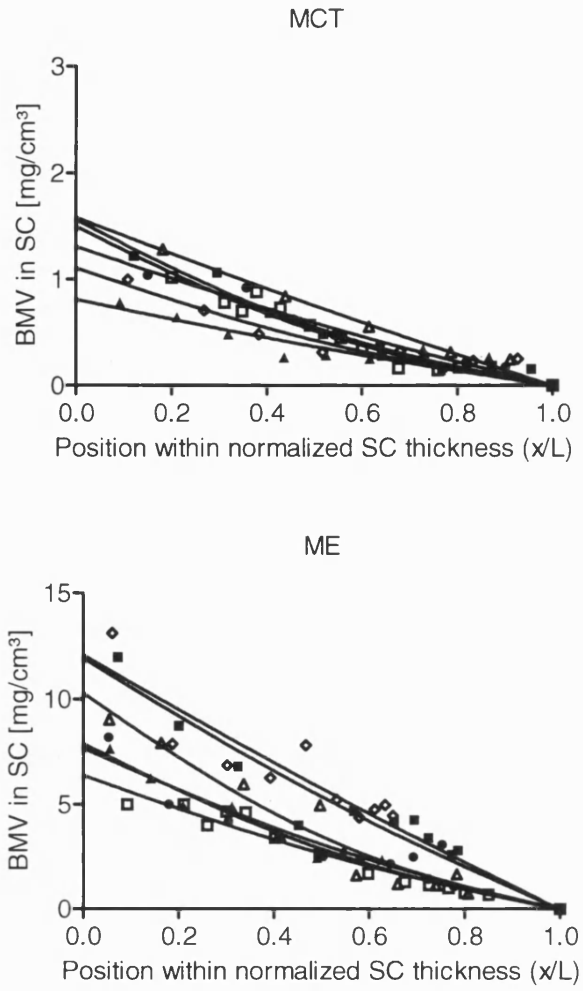


Figure 2: SC distribution profiles of BMV delivered from gelled MCT and ME formulations following a 6-hour application. The skin surface was cleaned by wiping with a dry paper towel. The best fits of Eq. 1 to the individual experimental data points are shown (n = 6).

Fitting the data to Eq. 1 allowed values of the partitioning (K) and diffusivity (D/L^2) parameters to be derived from the experiments performed after drug application times of 2 and 6 hours. These results and the AUCs, which were determined as described above, are collected in Table 1. It was also possible, using the K and D/L^2 parameters from the 2-hour experiments to predict, using Eq. 2, the AUCs anticipated following a longer application period of 6 hours. These calculations are also in Table 1.

Table 1: Partitioning and diffusivity parameters, as well as measured and predicted AUCs, and calculated saturation level of BMV in the SC, from the drug concentration profiles following application of gelled MCT and ME formulations to human volunteers (mean ± SD, n = 6).

Formulation	MCT	MCT	ME	ME
Application time [h]	2	6	2	6
K[a]	0.83 ± 0.24	0.77 ± 0.18	0.78 ± 0.10	1.01 ± 0.26
D/L^2 [h^{-1}][a]	0.058 ± 0.013	0.037 ± 0.013	0.056 ± 0.021	0.036 ± 0.012
$K \cdot C_{s,V} = C_{s,SC}$ (mg/ml)[b]	1.7 ± 0.5	1.6 ± 0.4	9.1 ± 1.2	11.8 ± 3.0
AUC_{expt} (mg/cm^3)[c]	0.51 ± 0.18	0.58 ± 0.14	2.61 ± 0.67	4.18 ± 1.27
AUC_{pred} (mg/cm^3)[d]	-	0.68 ± 0.21	-	3.38 ± 0.65

[a]Deduced from the fit of Eq. 1 to the experimental distribution profiles.

[b]$C_{s,SC}$ = calculated saturation level of BMV in the SC.

[c]Determined from Eq. 2 with the deduced values of K and D/L^2.

[d]Predicted from Eq. 2 using the values of K and D/L^2 deduced from the 2-hour data.

There were no significant differences (ANOVA, $P > 0.05$) between the deduced values of either K or D/L^2 for MCT and ME following either 2 or 6 hours of BMV delivery. However, there was a significant enhancement of drug uptake into the SC from ME relative to MCT: EF values were 5.6 (± 2.2) and 7.6 (± 2.9) at 2 and 6 hours, respectively. This was the result (see Table 1) of a significantly increased apparent saturation level of BMV in the SC caused, presumably, by the concomitant penetration of ME constituents into the barrier. This phenomenon has been reported previously for ibuprofen delivered from vehicles containing different proportions of propylene glycol and water [16,17]. The predicted AUCs at 6 hours based on the derived values of K and D/L^2 from the 2-hour experiments were in good agreement with the experimental findings for BMV uptake from both formulations. This confirms the potential of the technique, as has been proposed [18,19], to reduce the number of measurements necessary, for example, to establish bioequivalence between different drug products.

The results from the second set of experiments, in which the skin was more aggressively cleaned with an isopropyl alcohol swab, following a 2-hour

application of the gelled MCT and ME formulations, are in Figure 3. Similarly, Figure 4 presents the SC uptake profiles of BMV, when applied as un-gelled (liquid) formulations in the third series of studies; in this case, skin surface cleaning at the end of the administration period was accomplished with a dry paper towel.

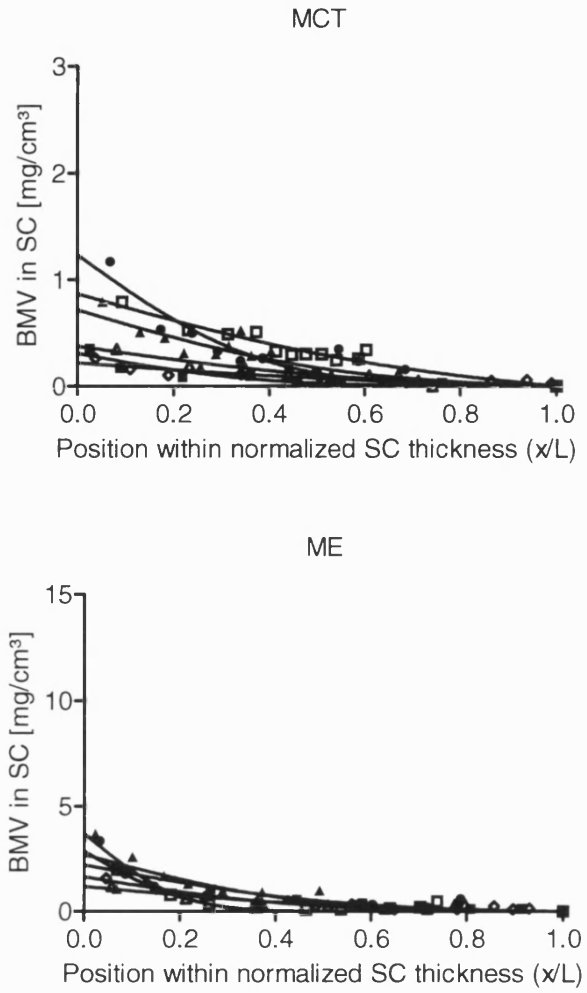


Figure 3: SC distribution profiles of BMV delivered from gelled MCT and ME formulations following a 2-hour application. The skin surface was cleaned using an isopropyl alcohol swab. The best fits of Eq. 1 to the individual experimental data points are shown (n = 6).

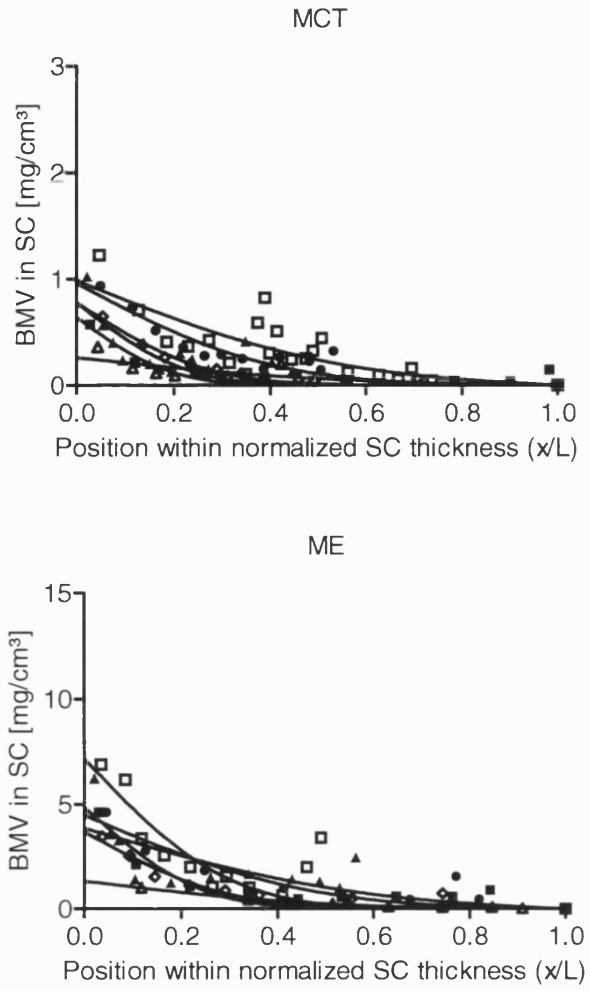


Figure 4: SC distribution profiles of BMV delivered from un-gelled MCT and ME formulations following a 2-hour application. The skin surface was cleaned with a dry paper towel. The best fits of Eq. 1 to the individual experimental data points are shown (n = 6).

Analysis of these two sets of results using Eqs. 1 and 2 generated the additional information (partitioning and diffusivity parameters, AUCs, saturation levels in the SC and EFs) collected in Table 2.

Table 2: Partitioning and diffusivity parameters, as well as measured AUCs, and calculated saturation levels of BMV in the SC and enhancement factors (EF), from the drug concentration profiles subsequent to a 2-hour application to human volunteers (mean ± SD, n = 6) of (i) gelled MCT and ME formulations followed by skin surface cleaning with isopropyl alcohol, and (ii) un-gelled vehicles followed by skin wiping with a dry paper towel.

Formulation	**MCT (i)**	**ME (i)**	**MCT (ii)**	**ME (ii)**
Experimental conditions	Isoprop.[a]	Isoprop.[a]	Un-gelled[b]	Un-gelled[b]
K[c]	0.37 ± 0.23	0.26 ± 0.10	0.43 ± 0.16	0.46 ± 0.20
D/L^2 [h^{-1}][c]	0.049 ± 0.023	0.028 ± 0.018	0.023 ± 0.018	0.026 ± 0.017
$K \cdot C_{s,V} = C_{s,SC}$ [mg/ml][d]	0.8 ± 0.5	3.0 ± 1.2	0.9 ± 0.3	5.3 ± 2.4
AUC_{expt} [mg/cm^3][e]	0.20 ± 0.12	0.54 ± 0.16	0.16 ± 0.10	0.97 ± 0.42
EF	-	4.0 ± 3.2[f]	-	6.9 ± 3.4[f]

[a]Skin surface cleaned using an isopropyl alcohol swab post-application of gelled vehicles.

[b]Skin surface cleaned using a dry paper towel post-application of un-gelled (liquid) vehicles.

[c]Deduced from the fit of Eq. 1 to the experimental distribution profiles.

[d]$C_{s,SC}$ = calculated saturation solubility of BMV in the SC.

[e]Determined from Eq. 2 with the deduced values of K and D/L^2.

[f]$EF = AUC_{ME}/AUC_{MCT}$.

It is immediately apparent that an isopropyl alcohol wipe of the skin surface alters the extent of apparent BMV delivery into the SC. AUCs were significantly reduced with respect to the values reported in Table 1. This was manifest in an approximately 50 % reduction in the SC-vehicle partition coefficients of the drug and a ~ 2-fold lowering in the deduced saturation levels of BMV in the SC. The values of D/L^2, on the other hand, were not significantly affected by the surface cleaning procedure, suggesting that the brief exposure of the skin served only to remove better sequestered formulation from skin 'furrows' and did not artefactually 'drive' drug into the SC. Nevertheless, the basic differences between BMV delivery from MCT and

ME formulations remained unchanged, with the latter still provoking a significant enhancement in uptake.

These conclusions were reinforced by the final experiments which considered BMV uptake into the SC from un-gelled, liquid formulations and skin cleaning with a dry paper towel. AUCs were again lower, as reflected in the lower values of K; in turn, these translated into reduced saturation levels in the SC. The order of magnitude of these changes were very similar to what had been seen when the gelled vehicles were removed with isopropyl alcohol. Clearly, the simpler, dry-wiping procedure is quite effective in removing material from deeper regions of the skin's surface topology for low-viscosity, liquid formulations; in contrast, a more aggressive approach is warranted for semi-solid products which are typically used in practice.

In summary, therefore, this research has provided further information essential for the evolution of the DPK, tape-stripping method as a tool for the measurement of bioavailability and bioequivalence. It is clear that quantitative comparisons between formulations must be performed 'on a level playing field' where it is certain that the SC uptake deduced from the analysis of the tape-strips is not contaminated by drug formulation trapped in skin 'furrows'. The careful evaluation and validation of an efficient skin cleaning procedure at the end of the application period is a pre-requisite.

4. Acknowledgements

We thank Sebapharma GmbH & Co. KG for providing the Mikro 100®. We are particularly grateful to Professor Annette Bunge for scientific critique, and to Dr. Christian Surber for the development and gift of equipment used in this work.

5. References

[1] Stinchcomb A. L., Pirot F., Touraille G. D., Bunge A. L., Guy R. H., Chemical uptake into human stratum corneum in vivo from volatile and non-volatile solvents, Pharm. Res. 1999; 16:1288-1293

[2] Tsai J.-C., Chuang S.-A., Hsu M.-Y., Sheu H.-M., Distribution of salicylic acid in human stratum corneum following topical application in vivo: a comparison of six different formulations, Int. J. Pharm. 1999; 188:145-153

[3] Pershing L. K., Silver B. S., Krueger G. G., Shah V. P., Skelley J. P., Feasibility of measuring the bioavailability of topical betamethasone dipropionate in commercial formulations using drug content in skin and a skin blanching assay, Pharm. Res. 1992; 9:45-51

[4] Shah V. P., Flynn G. L., Yacobi A., Maibach H. I., Williams R. L., et al., Bioequivalence of topical dermatological dosage forms-methods of evaluation of bioequivalence, Pharm. Res. 1998; 15:167-171

[5] Guidance for Industry: Topical dermatologic drug product NDAs and ANDAs-in vivo bioavailability, bioequivalence, in vitro release, and associated studies, Bethesda (MD): Center for Drug Evaluation and Research, FDA, 1998

[6] N'Dri-Stempfer B., Guy R. H., Navidi W. C., Bunge A. L. Dermatopharmacokinetic assessment of bioequivalence of topical 0.025% tretinoin gel products: Data re-analysis. In: Perspectives in Percutaneous Penetration; 2006; La Grande Motte; 2006. p. 26

[7] Fiedler M., Meier W. D., Hoppe U., Texture analysis of the surface of the human skin, Skin Pharmacol. 1995; 8:252-265

[8] Wiedersberg S., Leopold C. S., Guy R. H., Pharmacokinetics and dermatopharmacokinetics of betamethasone 17-valerate: Assessment of topical bioavailability, submitted for publication

[9] Kalia Y., Pirot F., Guy R., Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum in vivo, Biophys. J. 1996; 71:2692-2700

[10] Anderson R. L., Cassidy J. M., Variations in physical dimensions and chemical composition of human stratum corneum, J. Invest. Dermatol. 1973; 61:30-32

[11] Crank J. Diffusion in a plane sheet. In: Crank J, editor. The mathematics of diffusion. 2 ed. London: Oxford University Press; 1975. p. 44-68

[12] van der Molen R. G., Spies F., van 't Noordende J. M., Boelsma E., Mommaas A. M., Koerten H. K., Tape stripping of human stratum corneum yields cell layers that originate from various depths because of furrows in the skin, Arch. Dermatol. Res. 1997; 289:514-518

[13] Bashir S. J., Chew A.-L., Anigbogu A., Dreher F., Maibach H. I., Physical and physiological effects of stratum corneum tape stripping, Skin Res. Technol. 2001; 7:40-48

[14] Jacobi U., Meykadeh N., Sterry W., Lademann J., Effect of the vehicle on the amount of stratum corneum removed by tape stripping, J. Deut. Dermatol. Gesell. 2003; 1:884-889

[15] Kalia Y. N., Alberti I., Sekkat N., Curdy C., Naik A., Guy R. H., Normalization of stratum corneum barrier function and transepidermal water loss in vivo, Pharm. Res. 2000; 17:1148-1150

[16] Herkenne C., Naik A., Kalia Y. N., Guy R. H., Effect of propylene glycol on ibuprofen absorption into human skin in vivo, submitted for publication

[17] Herkenne C., Naik A., Kalia Y. N., Hadgraft J., Guy R. H., Pig ear skin ex vivo as a model for in vivo dermatopharmacokinetic studies in man, Pharm. Res. 2006; 23:1850-1856

[18] Alberti I., Kalia Y. N., Naik A., Guy R. H., Assessment and prediction of the cutaneous bioavailability of topical terbinafine, in vivo, in man, Pharm. Res. 2001; 18:1472-1475

[19] Herkenne C., Naik A., Kalia Y. N., Hadgraft J., Guy R. H., Dermatopharmacokinetic prediction of topical drug bioavailability in vivo, J. Invest. Dermatol. In Press

CHAPTER 5

Effect of various vehicles on skin hydration in vivo

Effect of various vehicles on skin hydration in vivo

Sandra Wiedersberg[1], Claudia S. Leopold[2], Richard H. Guy[1]

[1]Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK.

[2]Institute for Pharmacy, Department of Pharmaceutical Technology, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany.

Short communication to be submitted

1. Introduction

The stratum corneum (SC) is the outermost layer of the epidermis and is primarily responsible for skin's barrier function [1]. In particular, the SC regulates the passive loss of water to the environment [2]. The SC consists of more or less parallel layers of proteinaceous corneocytes, interspersed with hydrophobic intercellular lipid bilayers [3]. The intercellular SC lipids are primarily ceramides (~ 40 % w/w), free fatty acids (~ 10 % w/w), and cholesterol (~ 25 % w/w), with a small fraction of cholesterol sulphate and triglycerides [4,5]. The SC lipids are believed to be responsible for regulating the passive flux of water through the SC from the deeper, highly hydrated layers of the epidermis to the surface of the barrier where the moisture content is relatively low [6].

On average, water in the SC corresponds to 15 - 20 % of the dry weight, but varies according to humidity of the external environment [7]. The water content of the SC comprises both that bound in corneocytes and that associated with the intercellular lipids. Measurement of skin hydration has been used to assess barrier function integrity *in vivo* [8]. It is also well-known that drug penetration can be influenced by skin hydration, which may be increased via physical occlusion or by the application of moisturizing or oleaginous vehicles [9]. Increased SC hydration may alter partitioning of a drug from a formulation and/or the solubility of the drug in the barrier. Measurements of transepidermal water loss (TEWL) or of skin hydration are useful approaches with which to assess vehicle effects on SC barrier function. The latter employs a so-called corneometer which provides a relative assessment of skin surface (SC) hydration.

In an earlier *in vivo* study, the skin blanching and tape-stripping methods were used to assess the penetration of betamethasone 17-valerate (BMV) from different vehicles [10]. Specifically, a microemulsion, light mineral oil and a formulation based on the penetration enhancer, Transcutol®, were compared with a reference vehicle which comprised medium chain triglycerides (MCT). The tape-stripping results showed that Transcutol® and the microemulsion significantly enhanced the penetration of BMV relative to that from MCT.

The aims of this pilot investigation, therefore, were to evaluate the effect of these same vehicles on skin hydration and to compare the results with those from the topical availability measurements.

2. Materials and methods

2.1. Preparation of the formulations

The vehicles investigated were: light mineral oil (LMO), medium chain triglycerides (MCT) (Synopharm, Barsbüttel, Germany), the microemulsion Mikro 100® (ME) (Sebapharma, Boppard, Germany) and diethyleneglycol monoethyl ether (Transcutol®, TCL) (Gattefossé, Saint Priest, France). LMO and MCT were gelled with 15 % polypropylene, ME and TCL with 10 % Aerosil 200® (both Sigma-Aldrich, Steinheim, Germany).

2.2. *In vivo* experiments

Eight healthy, female volunteers (21 - 29 years) participated in the study, which was approved by the Ethics Committee of the University of Leipzig. MCT was chosen as the reference vehicle because it was not expected to exert appreciable effects on the skin [11]. A single, infinite dose comprising 250 µl of each formulation, was applied via a 1.2 cm diameter Hill Top Chamber® (Hill Top Research Inc., Cincinnati, OH, USA) to the volar aspect of the forearm for different dose durations of up to 6 hours. At the end of the treatment period, the chambers were removed and excess formulation was gently wiped away using a dry paper towel. The amount of moisture in the outer layer of the skin (skin hydration) was measured before application, immediately after removing the formulation, and then repeatedly over the next 40 minutes. The latter was accomplished using a corneometer CM 825® (Courage & Khazaka, Cologne, Germany) (Figure 1) mounted on a Multi Probe Adapter® MPA 5 (Courage & Khazaka). The apparatus determines the relative capacitance of the skin, to a measurement depth of approximately 10-20 µm, via a probe that consists of two closely juxtapositioned, finger-type metal plates. Changes in the water content of the SC, therefore, cause a change in the local dielectric constant and hence a proportional change (expressed in arbitrary units) in capacitance. A spring in the probe head ensures that the latter is reproducibly applied at a constant pressure to the skin. At separate skin sites pre- and post-treatment (at 2 hours for all formulations, and at 6 hours for MCT and ME), TEWL was measured

(AquaFlux V4.7, Biox Systems Ltd., London, UK). Five individual measurements were performed on each tested skin site and the average value was determined. All experiments were carried out under reasonably similar conditions of relative humidity (40-60 %) and temperature (20-25 °C).

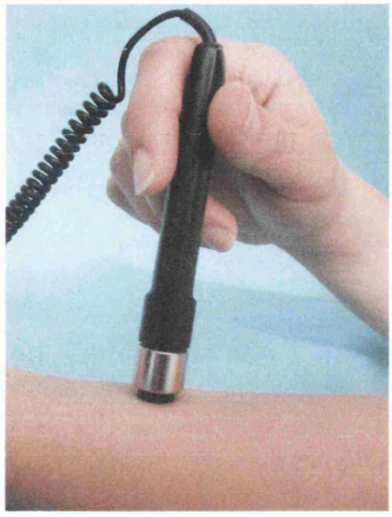


Figure 1: Skin hydration measured with the corneometer.

2.3. Statistics

Analysis of variance was performed using PRISM 4.01 (GraphPad, San Diego, LA). Changes over time with different vehicles were compared with a two-way ANOVA followed by an α-adjusted post-hoc Bonferroni test. Vehicle-dependent changes were assessed with an one-way ANOVA and a Dunnett post-test.

3. Results and discussion

To facilitate comparison between vehicles, the absolute values of skin hydration were normalized with respect to the corresponding pre-treatment control measurements. The variations of these normalized results, as a function of dose duration, are in Figure 2. All vehicles produced a significant increase in skin hydration versus the untreated control. Constant levels of hydration were reached after 30 minutes application of all vehicles. Extending the dose duration up to 6 hours did not elicit any further change in skin hydration. ME significantly increased skin hydration relative to MCT after 30 minutes; on the other hand, TCL significantly lowered skin hydration (again, relative to MCT) after a 4-hour application. In a brief parallel experiment (dose duration = 30 minutes), it was shown that the elimination of the gelling agent did not alter the findings reported in any way (data not shown).

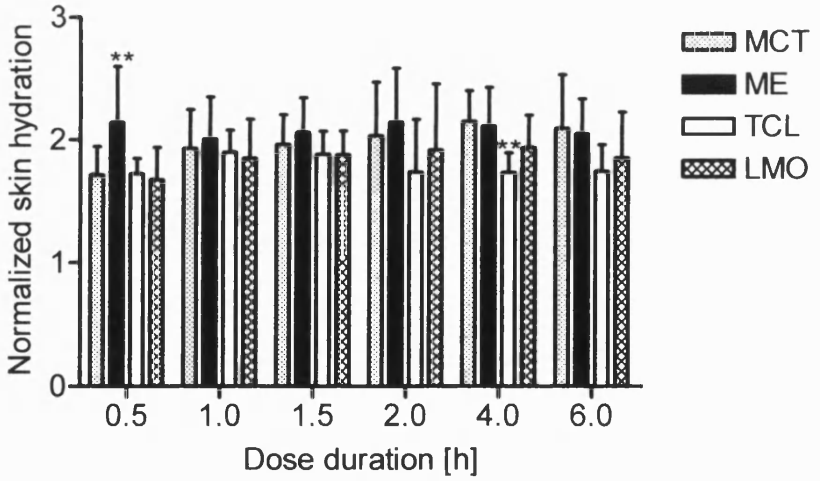


Figure 2: Normalized skin hydration as a function of exposure time to four different vehicles (mean ± SD, n = 8). Significant differences ($P < 0.01$) relative to the reference formulation (MCT) are shown by the double asterisks.

Recoveries of skin hydration post-removal of the formulations (after an exposure duration of 2 hours) are in Figure 3. The initially high values diminish quite rapidly to the control level (normalized skin hydration = 1); for TCL, however, the decrease was significantly greater, with the normalized

skin hydration clearly dropping to below 1. It would appear, therefore, that TCL is able to draw water from the SC, an effect which may explain its deduced ability to increase the solubility of the lipophilic betamethasone 17-valerate in the SC [10]. In contrast, the ME vehicle, which also improved the steroid's uptake into the SC (albeit to a much lower extent than TCL) [10,12], did not appear to elicit any long-term effect on skin hydration, at least none that could be detected at the sensitivity of the corneometer measurements.

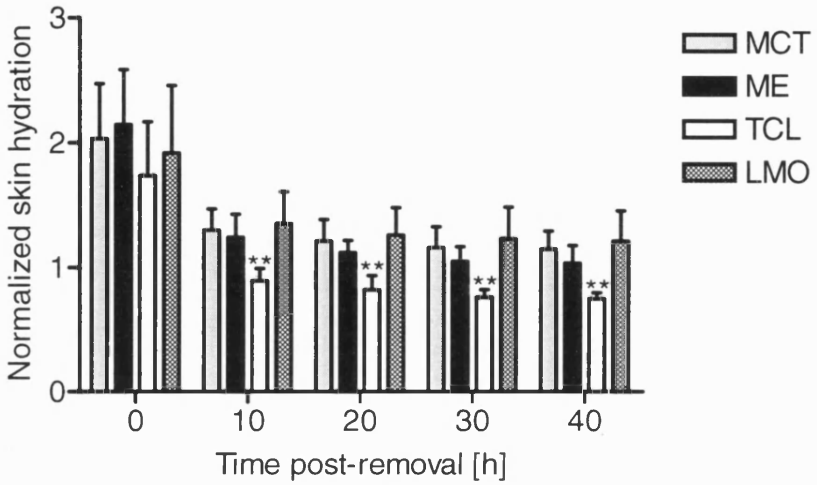


Figure 3: Normalized skin hydration as a function of time post-removal of different vehicles applied for 2 hours (mean ± SD, n = 8). Significant differences ($P < 0.01$) relative to the reference formulation (MCT) are shown by the double asterisks.

These conclusions are confirmed by the TEWL results in Figure 4. While occlusion for 2 or 6 hours with the MCT and ME formulations caused a marked increase in TEWL immediately post-removal (relative to the pre-treatment control), the effect of TCL was significantly less, effectively obliterating the impact of the occlusion period of exposure to the vehicle. Again, this finding supports the idea that TCL draws water from the SC.

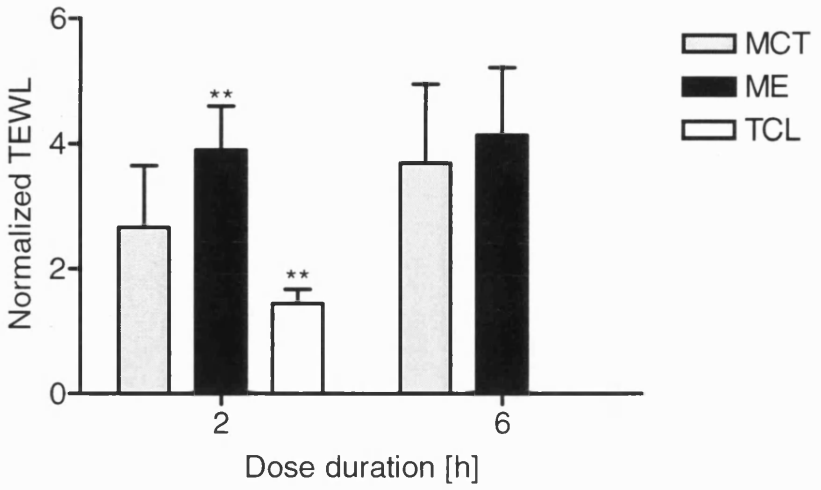


Figure 4: Normalized TEWL values (post-removal divided by pre-treatment) immediately after removal of the different formulations tested (mean ± SD, n = 6). Significant differences ($P < 0.01$) relative to the reference formulation (MCT) are shown by the double asterisks.

4. References

[1] Scheuplein R. J., Blank I. H., Permeability of the skin, Physiol. Rev. 1971; 51:702-747

[2] Golden G. M., Guzek D. B., Kennedy A. E., McKie J. E., Potts R. O., Stratum corneum lipid phase transitions and water barrier properties, Biochemistry 1987; 26:2382 - 2388

[3] Wertz P. W., The nature of the epidermal barrier: biochemical aspects, Adv. Drug Delivery Rev. 1996; 18:283-294

[4] Elias P. M., Epidermal barrier function: intercellular lamellar lipid structures, origin, composition and metabolism, J. Controlled Release 1991; 15:199-208

[5] Wertz P. W., Downing D. T. Stratum corneum: Biological and biochemical considerations. In: Hadgraft J, Guy RH, Editors. Transdermal Drug Delivery. New York: Marcel Dekker; 1989. p. 1-22

[6] Simonetti O., Hoogstraate A. J., Bialik W., Kempenaar J. A., Schrijvers A. H. G. J., Bodde H. E., et al., Visualization of diffusion pathways across the stratum corneum of native and in-vitro-reconstructed epidermis by confocal laser scanning microscopy, Arch. Dermatol. Res. 1995; 287:465-473

[7] Benson H. A. E., Transdermal drug delivery: Penetration enhancement techniques, Curr. Drug Deliv. 2005; 2:23-33

[8] Rim J. H., Jo S. J., Park J. Y., Park B. D., Youn J. I., Electrical measurement of moisturizing effect on skin hydration and barrier function in psoriasis patients., Clin. Exp. Dermatol. 2005; 30:409-413

[9] Zhai H., Maibach H. I., Occlusion vs. skin barrier function, Skin Res. Technol. 2002; 8:1-6

[10] Wiedersberg S., Leopold C. S., Guy R. H., Pharmacokinetics and dermatopharmacokinetics of betamethasone 17-valerate: Assessment of topical bioavailability, submitted for publication

[11] Leopold C. S., Lippold B. C., Enhancing effects of lipophilic vehicles on skin penetration of methyl nicotinate in vivo, J. Pharm. Sci. 1995; 84:195-198

[12] Wiedersberg S., Naik A., Leopold C. S., Guy R. H., Topical application of betamethasone 17-valerate: Tape-stripping versus pharmacodynamic response, submitted for publication

Conclusions

Despite the importance and frequent use of topical glucocorticoids in dermatology, their topical bioavailability (BA) and bioequivalence (BE) remains difficult to assess. While it is generally agreed that corticosteroid BA is poor, a quantitative measure of this key parameter has proven elusive. The objective of this thesis, therefore, was to evaluate the potential of two non-invasive, *in vivo* techniques to determine the topical BA of a topical steroid. The first, the vasoconstrictor assay, is already accepted by regulatory agencies, such as the U.S. Food and Drug Administration (FDA), for BA/BE testing. This method is based on the ability of topical glucocorticoids to cause visible and quantifiable skin blanching. The second method is the dermatopharmacokinetic (DPK) approach using tape-stripping, which is being intensively investigated at this time. Betamethasone 17-valerate (BMV) was selected as a model corticosteroid and was formulated in several vehicles at different levels of saturation.

The vasoconstrictor assay may, in the right circumstances, be a useful method to determine the local BA of topical glucocorticoids. The results of the vasoconstrictor assay performed with BMV delivered from different vehicles, and over a range of different concentrations, resulted in a clear concentration dependence of the skin blanching response until saturation occurred (Figure 1). Subsequently, no further differences between formulations or concentrations applied could be detected. As with any pharmacodynamic response, this is an potential issue and it is important, therefore, to operate in the linear part of the 'dose-response' curve whenever quantitative conclusions about BA/BE are to be drawn. However, this linear part of the 'dose-response' curve for BMV appeared to be narrow and the variability of the pharmacodynamic response was quite high (40 - 60 %); it follows that a large number of subjects is needed to identify statistically significant differences. It is clear that the vasoconstrictor assay remains some way from an absolute measurement of the extent of drug delivery to the skin (at or near the target site) and the rate at which this is achieved. Further, it is obviously

limited to drugs which elicit an easily detectable and quantifiable pharmacological response.

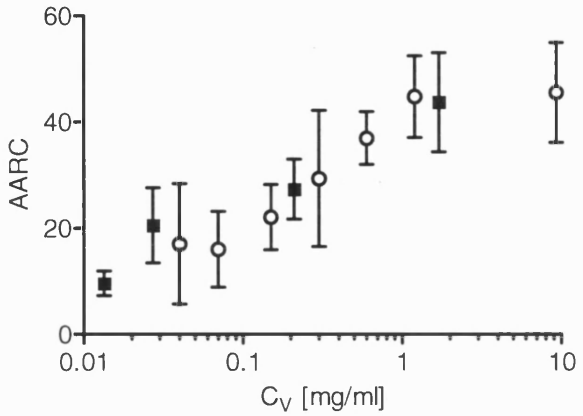


Figure 1: Skin blanching response (expressed as AARC) as a function of BMV concentration applied in two different vehicles (C_V). Mean ± SD, n = 6.

The dermatopharmacokinetic (DPK) approach, using tape-stripping, appeared to offer a reliable metric with which to quantify the effective amount of drug penetrating into the major barrier for percutaneous absorption, the stratum corneum (SC). This technique has the advantage of being applicable to a broader range of topical drug classes; it is also simple and relatively non-invasive. In 1998, the FDA proposed a Draft Guidance, analogous to the pharmacokinetic method of oral drug BA/BE assessment, whereby the drug uptake and elimination into and from the SC were to be determined as a function of time post-application and post-removal. Although the DPK profiles of BMV according to the Draft Guidance showed reasonable reproducibility and a clear ability to distinguish between different formulations, some clear weaknesses have been exposed: specifically, the similar design of the approach to oral BA assessment makes the procedure rather labour-intensive, and the lack of quantification of the SC amount removed is problematic due to inter-subject variability in the thickness and cohesivity of this membrane.

As a consequence, a critical re-evaluation of the DPK method is in progress, with a clear objective to validate the method. Important progress has already been made with regard to quantification and standardization of the amount of SC removed using tape-stripping such that drug concentration profiles across the SC from different volunteers can now be expressed on the same scale: that is, as a function of relative position within the SC. Equally, dermatopharmacokinetic parameters, characterizing drug partitioning and diffusivity into and through the SC, can be deduced and used to quantify, respectively, the extent and rate of drug delivery. The normalized distribution profiles of BMV across the SC after a 2-hour uptake were, like the DPK profiles, sensitive enough to discriminate between formulations and different concentrations applied.

The cleaning procedure before tape-stripping has also to be validated to ensure that the residual formulation on the skin surface is completely removed and that the amount analysed from the tape-strips includes only the amount of drug which has penetrated into the SC rather than that which may be deposited in skin 'furrows'. The cleaning procedure, therefore, must be efficient, but not so aggressive to 'drive' compounds into the SC nor to extract it therefrom. It was shown in this thesis that wiping the skin before stripping with an isopropyl alcohol swab was much more efficient than cleaning with a dry paper towel (as recommended by the FDA). This more aggressive cleaning procedure led to a significant decrease (~ 2-fold) in the apparent extent of drug delivery into the SC and in the partition coefficient. Excess formulation, especially from semi-solids, may be trapped in the skin 'furrows' and could lead to a overestimation of the apparent extent of drug delivery into the SC, if not removed completely. A careful evaluation and validation of an efficient skin cleaning procedure at the end of the application period is, therefore, a pre-requisite. Also the viscosity of the formulation was shown to influence the amount of drug recovered on the tape-strips. Liquid formulations can be removed more easily from the skin's surface with a dry-wiping procedure, whereas a more aggressive approach is warranted for semi-solid vehicles (Figure 2).

Other techniques, such as optical microscopy or attenuated total reflectance Fourier transform infrared spectroscopy, may also prove useful for better characterizing the deposition of a formulation and its delivered drug (and the efficiency of a cleaning process) on the skin surface. Future experiments may usefully apply these methods to complement the work already done.

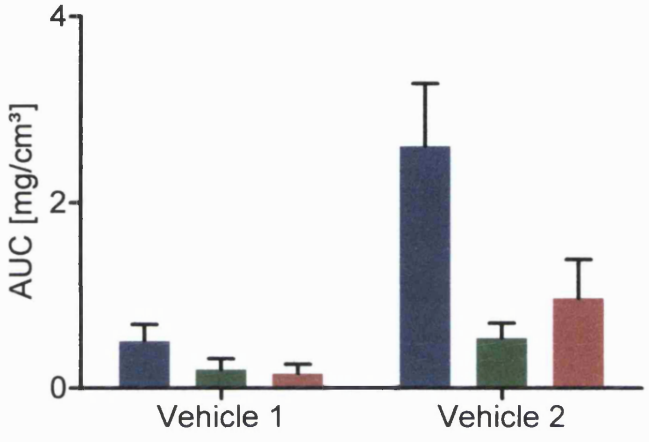


Figure 2: Apparent extent of BMV delivery in the SC (expressed as AUC) from two vehicles as a function of the cleaning procedure before tape-stripping. Semi-solids cleaned with a dry paper towel (blue), semi-solids cleaned with an isopropyl alcohol swab (green), liquids cleaned with a dry paper towel (red). Mean ± SD, n = 6.

A weakness of the DPK approach is that the measurement 'compartment' is not always the same as the site of action. It has to be assumed, that SC drug levels are directly related to those in the deeper skin layers. For steroids, the target receptors are within the viable epidermis and dermis, not in the SC. Nevertheless, it seems likely that a formulation, which changes drug uptake into the SC, which is the major barrier to steroid absorption, should also change the drug's BA at the site of action. The permeation/diffusion of the drug then from the SC into the deeper skin layers should be independent of the vehicle and a function primarily of the properties of the drug itself (lipophilicity, receptor affinity, etc.).

An alternative approach, already subject to serious evaluation, and one certainly worthy of further consideration and study, is microdialysis. While serious technical challenges remain, this method does allow topical drug concentrations in regions of the skin close to the site(s) of drug action to be determined.

Overall, however, it may be argued that the tape-stripping method, while not yet completely optimised, and perhaps more complicated than the vasoconstrictor assay, is nevertheless straightforward and relatively easy to perform. This technique is sensitive enough to differentiate between formulations and between concentrations. With further development, the DPK approach may offer a more quantitative and objective strategy for topical BA assessment of topical glucocorticoids than the vasoconstrictor assay.

Finally, it is worth considering what else may be necessary to enhance further the use of the DPK procedure for routine topical BA/BE assessment? The most onerous and time-consuming part of the method is the careful weighing of each tape-strip before and after SC removal to determine the amount of tissue removed. The gravimetric approach is not without problems (static electricity on the tapes, small amounts of SC removed, etc.) and a more direct and simple assessment of the amount of SC removed would be useful. A spectrophotometric method is one alternative, and is based on the determination of the absorbance (scattering, reflection and diffraction) of the corneocyte aggregates, attached to the tape, in the visible spectral range. This technique also has the potential to quantify the drug directly on the tape-strips if it has an absorbance clearly separated from those of the SC. Indeed, there have already been some promising correlations observed between the weight of SC removed and the absorbance at 430 nm of SC stripped off with Tesa tape (No. 5529, Beiersdorf AG, Hamburg, Germany) [1,2]. To-date, however, this approach has not been fully optimised or characterized.

Some preliminary experiments are therefore been performed to evaluate the suitability of the spectrophotometric method to assess the SC amount removed using the methods described in this thesis. Scotch Book

tape 845 (3M, St. Paul, MN, USA) is more adhesive than Tesa tape. In a pilot study, the untreated SC of 13 volunteers was tape-stripped and was quantified either gravimetrically or by measuring the absorbance spectrophotmetrically. On average 20 tape-strips per volunteer were taken to ensure that at least 75 % of the SC had been removed (as confirmed by transepidermal water loss measurements). The amount of corneocytes adhering to each tape was quantified directly on the tape at 430 nm (Lambda 35 spectrophotometer, Perkin Elmer, Überlingen, Germany) with an empty tape as reference.

Figure 3 shows the UV/VIS spectra of a tape-strip removed from the forearm and a blank tape without any SC attached. As the latter indicates essentially is no absorbance at 430 nm (as described in the literature for Tesa tape as well), the same wavelength was used to determine the amount of SC removed by the Scotch Book tape used in this work.

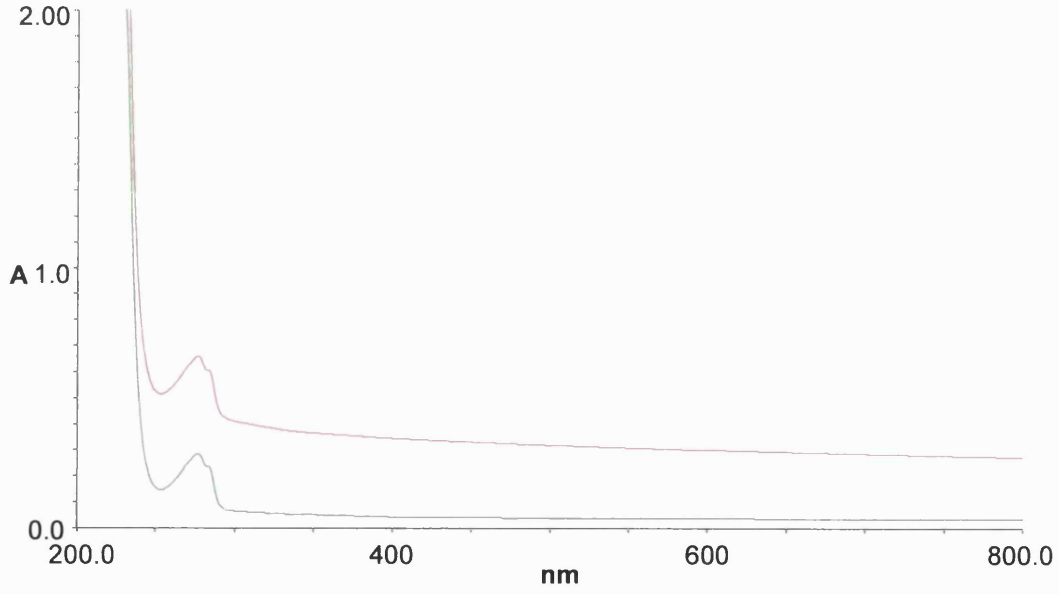


Figure 3: UV/VIS spectra of a tape-strip without (green line) and with SC (red line).

Absorbance at 430 nm was plotted against the thickness of the corneocyte aggregates removed on each tape-strip (Figure 4) and linear regressions were performed on the results from each subject (Table 1).

Absorbance increased with the thickness of SC removed, and almost all correlations were significant (11 out of 13 have $R^2 \geq 0.69$).

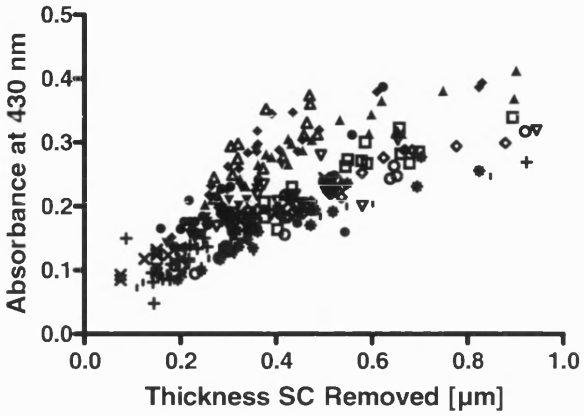


Figure 4: Absorbance at 430 nm versus thickness of SC layer removed on each tape-strip. Data comprise 256 pooled readings from 13 volunteers.

Table 1: Calculated slopes, y-intercepts and R^2 values following linear regression of the datasets from each volunteer.

Volunteer	Slope (μm^{-1})	y-intercept	R^2
1	0.56	0.03	0.92
2	0.25	0.10	0.71
3	0.30	0.12	0.88
4	0.35	0.06	0.86
5	0.36	0.06	0.89
6	0.32	0.05	0.90
7	0.28	0.19	0.40
8	0.18	0.14	0.25
9	0.27	0.16	0.86
10	0.36	0.14	0.73
11	0.23	0.07	0.69
12	0.25	0.05	0.96
13	0.29	0.05	0.87
Mean	**0.31**	**0.09**	-
SD	**0.09**	**0.05**	-

Nevertheless, there is clearly important variability between subjects, in terms of the slopes obtained. It would not be possible, therefore, at this time, to define a single proportionality constant with which to convert absorbance to a SC thickness. Intra-subject variability has not yet been determined but clearly represents an important next step in the evaluation of this approach.

Some effort was made to examine whether the observed variability was due to inhomogeneity in the distribution of SC across the surface of the tape-strip. The total area stripped in these experiments was 4 cm^2, while the spectrophotometer beam only interrogates the central 1 cm^2 of the sample. Consideration of a random selection of tapes revealed that the apparent inhomogeneity accounted for less than 8 % of the variability, insufficient to fully explain the results, therefore.

Additional work is obviously needed and, again, microscopic examination may be helpful in asking whether the more aggressive Scotch adhesive removes more than one SC layer per strip in some subjects. It is also sensible to re-consider the value of a more direct, chemical quantification (i.e., protein assay [3-5]) of the SC on the tape-strips. The latter should ultimately be amenable to high-throughput screening, a clear advantage if the DPK method is to be used routinely.

References:

[1] Weigmann H.-J., Lademann J., Meffert H., Schaefer H., Sterry W., Determination of the horny layer profile by tape stripping in combination with optical spectroscopy in the visible range as a prerequisite to quantify percutaneous absorption, Skin Pharmacol. Appl. Skin Physiol. 1999; 12:34-45

[2] Weigmann H.-J., Lindemann U., Antoniou C., Tsikrikas G. N., Stratigos A. I., Katsambas A., et al., UV/VIS absorbance allows rapid, accurate, and reproducible mass determination of corneocytes removed by tape stripping, Skin Pharmacol. Appl. Skin Physiol. 2003; 16:217-227

[3] Lindemann U., Weigmann H.-J., Schaefer H., Sterry W., Lademann J., Evaluation of the pseudo-absorption method to quantify human stratum corneum removed by tape stripping using protein absorption, Skin Pharmacol. Appl. Skin Physiol. 2003; 16:228-236

[4] Bashir S. J., Chew A.-L., Anigbogu A., Dreher F., Maibach H. I., Physical and physiological effects of stratum corneum tape stripping, Skin Res. Technol. 2001; 7:40-48

[5] Dreher F., Arens A., Hostynek J. J., Mudumba S., Ademola J., Maibach H. I., Colorimetric method for quantifying human stratum corneum removed by adhesive-tape-stripping, Acta Derm. Venereol. 1998; 78:186-189

List of publications

(*) Appear in this thesis

Papers

- Wiedersberg S., Leopold C.S., Guy R.H.. Bioavailability and bioequivalence of topical glucocorticoids. (*)
  To be published
- Wiedersberg S., Naik A., Leopold C.S., Guy R.H.. Topical application of betamethasone 17-valerate formulations: Tape-stripping versus pharmacodynamic response. (*)
  To be published
- Wiedersberg S., Leopold C.S., Guy R.H.. Pharmacodynamics and dermatopharmacokinetics of betamethasone 17-valerate: Assessment of topical bioavailability. (*)
  To be published
- Wiedersberg S., Leopold C.S., Guy R.H.. Dermatopharmacokinetics of betamethasone 17-valerate: Influence of formulation viscosity and skin surface cleaning procedure. (*)
  To be published
- Wiedersberg S., Leopold C.S., Guy R.H.. Effect of various vehicles on skin hydration in vivo. (*)
  To be published

Posters

- Wiedersberg S., Leopold C.S.. Evaluation of the enhancing effect of a microemulsion with the skin blanching assay.
  Annual Meeting of the German Pharmaceutical Society, Wuerzburg, Germany, October 8 - 11, 2003

- Wiedersberg S., Leopold C.S.. Influence of penetration kinetics on the determination of the relative bioavailability of corticosteroid formulations with the skin blanching assay.
  International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Nuremberg, Germany, March 15 - 18, 2004
- Wiedersberg S., Remane Y., Leopold C.S.. Quantification of the cutaneous betamethasone-17-valerate and methyl nicotinate penetration according to the FDA Guidance "Topical Dermatologic Corticosteroids".
  31st Annual Meeting of the Controlled Release Society, Honolulu, USA, June 12 - 16, 2004
- Wiedersberg S., Naik A., Leopold C.S., Guy R.H.. Dermatopharmacokinetic (DPK) assessment of topical formulations of betamethasone-17-valerate (BMV).
  Annual Meeting of the American Association of Pharmaceutical Scientists, Baltimore, USA, November 7 - 11, 2004
- Wiedersberg S., Leopold C.S., Guy R.H.. Assessment of the stratum corneum (SC) distribution profile of betamethasone-17-valerate (BMV) from different vehicles.
  6th Annual Meeting of Skin Forum, Winchester, UK, June 29 – July 1, 2005
- Wiedersberg S., Leopold, C.S., Guy R.H.. Dermatopharmacokinetics of betamethasone-17-valerate: Influence of the drug removal procedure.
  5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Geneva, Switzerland, March 27 – 30, 2006
- Wiedersberg S., Leopold, C.S., Guy R.H.. Dermatopharmacokinetics of betamethasone-17-valerate: Influence of drug thermodynamic activity.
  7th Annual Meeting of Skin Forum, Sheffield, UK, June 21 – 23, 2006

- Wiedersberg S., Leopold, C.S., Guy R.H.. Dermatopharmacokinetics of betamethasone-17-valerate: Influence of the gelling agent.
  APGI Skin and Formulation 2nd Symposium, Versailles, France, October 9 - 10, 2006

Podium presentations

- Wiedersberg S., Leopold C.S.. A pharmacokinetic model for analysis of the pilot study described in the FDA Guidance „Topical Dermatologic Corticosteroids: In Vivo Bioequivalence".
  30th Annual Meeting of the Controlled Release Society, Glasgow, United Kingdom, July 19 - 23, 2003
- Wiedersberg S., Remane Y., Leopold C.S.. Quantifizierung der kutanen Penetration von Betamethasone-17-valerat und Methylnicotinat durch Wirkungsmessungen.
  1. Treffen der Pharmazeutischen Technologie Mitteldeutschlands, Halle/Saale, Germany, October 22nd, 2004

Previous publications

Papers

- Fauth C., Wiedersberg S., Neubert R.H.H., Dittgen M.. Adhesive backing foil interactions affecting the elasticity, adhesion strength of laminates, and how to interpret these properties of branded transdermal patches.
  Drug Development and Industrial Pharmacy (2002), 28:1251-1259.

Posters

- Wiedersberg S., Fauth C., Neubert R.H.H., Dittgen M.. The 180° peel adhesion test - a real measure of transdermal drug delivering patches skin adhesion?.
  4th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Florence, Italy, April 8 - 12, 2002

- Fauth C., Wiedersberg S., Neubert R.H.H., Dittgen M.. Adhesion energies - a suitable measure to peel and skin adhesion for assessment of transdermal active patches?.
  4th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Florence, Italy, April 8 - 12, 2002
- Wiedersberg S., Fauth C., Neubert R.H.H., Dittgen M.. Evaluation of mouse skin to determine peel adhesion and work of adhesion of branded transdermal patches and correlation of the results to skin adhesion.
  1st EUFEPS Conference on Optimising Drug Delivery and Formulation: New Challenges in Drug Delivery, Versailles, France, September 29 - October 1, 2003

Appendix

1. Analytical method

BMV in the various samples was quantified by HPLC (Dionex, Sunnyvale, CA, USA) with UV detection at 240 nm using a Lichrospher® 100 RP-18 column (4 x 125 mm) and pre-column (Hichrom, Reading, UK). The mobile phase was degassed acetonitrile/water (60:40 v/v) delivered at a flow rate of 1 ml/min in a 50-µl sample loop. The retention time of BMV at 25 °C was ~ 3.8 minutes. A calibration curve was generated using solutions of the pure compound at 5 different concentrations (Figure 1). The detection limit was 0.03 µg/ml. The quantification limit was 0.1 µg/ml.

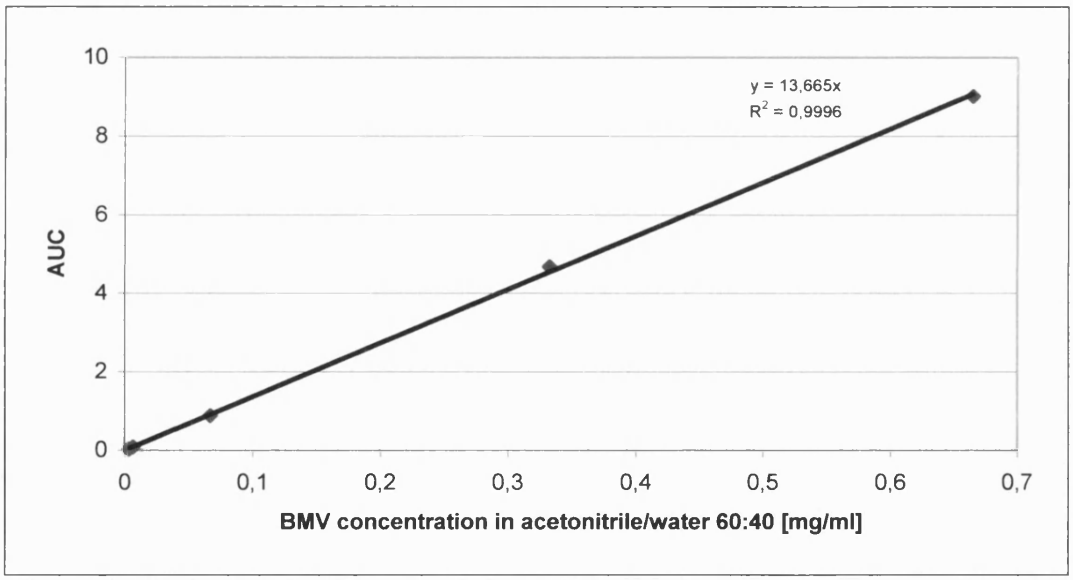


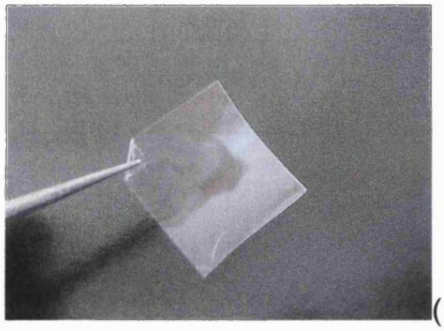
Figure 1: Typical calibration curve for BMV.

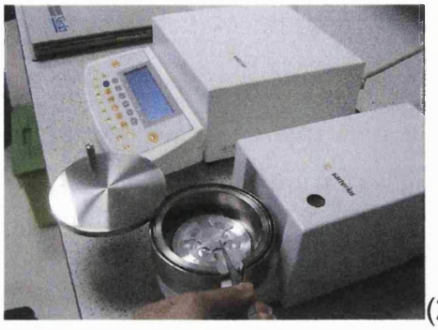
2. Tape-stripping protocol

A maximum of three application sites were selected on the forearm of a healthy volunteer. The area of each site was approximately 4 cm^2. One of these application sites was used for the determination of the total stratum corneum (SC) thickness; the other two were used for the application of the formulations and the determination of the SC drug profiles.

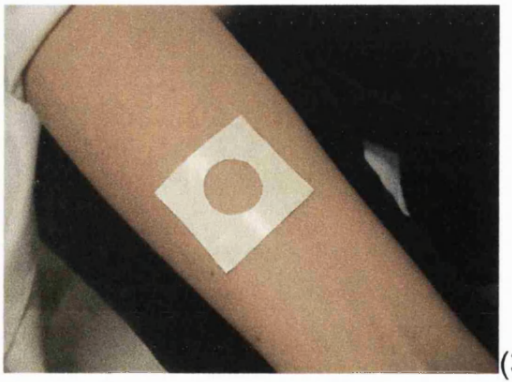
Site A: Determination of the total SC thickness

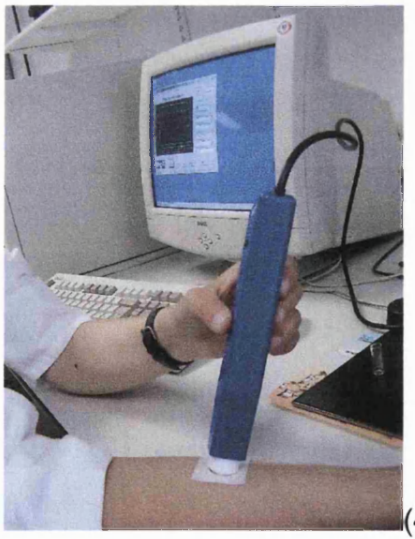
Approximately 25 tapes (Scotch® No. 845 Book Tape, 3M, St. Paul, MN, USA) were cut into pieces of 2.5 x 2.5 cm² (1) and allowed to equilibrate for at least 12 hours in the laboratory. The weight of each tape was determined using a balance with a precision of at least 10-µg (2).


(1)


(2)

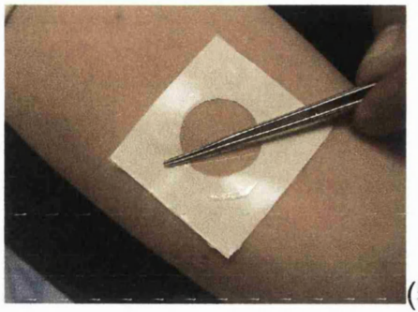
A template (piece of polypropylene foil), into which a 2.0 cm diameter hole had been cut, was affixed onto an untreated skin site (3). This template ensured a constant skin area. The initial transepidermal water loss (TEWL) of this skin area was measured (4) (AquaFlux V4.7, Biox Systems Ltd., London, UK).

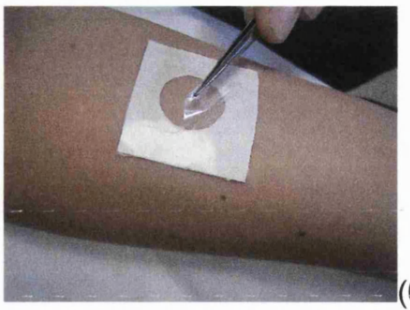

(3)


(4)

Subsequently, the SC was progressively removed by repeated adhesive tape-stripping. The pre-cut tape was applied to the template (5), pressed down 10-times with a constant pressure (140 g/cm²) using a weighted roller and then removed with one quick movement (6). The TEWL was measured after each tape-strip removed. Tape-stripping was continued

until the TEWL reached 4-fold the initial value. This was to ensure, that the SC was never stripped completely but at least 75 % of the SC was removed.


(5)


(6)

Each tape was re-weighed after stripping to assess the mass of SC removed. From this mass, and knowing the stripping area and the density of the SC (1g/cm³), it was possible to calculate the thickness of SC removed with each tape. The total thickness of the SC was then calculated from the x-axis intercept of a graph of 1/TEWL versus the cumulative thickness of SC removed (Figure 2).

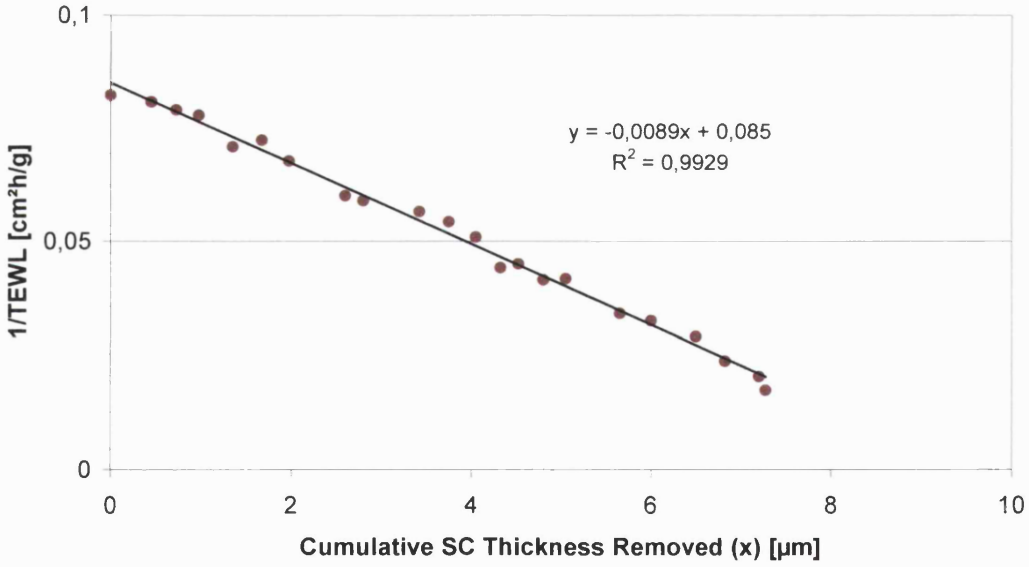
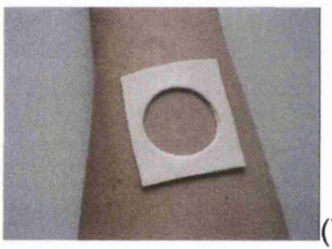
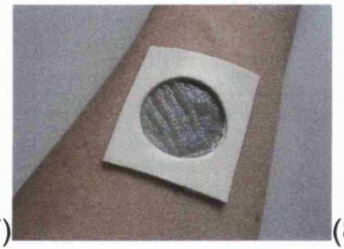
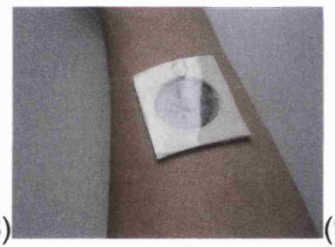


Figure 2: Plot of 1/TEWL versus cumulative SC thickness removed from a volunteer. Total thickness of the SC equals 9.55 µm.

Site B: Application of a formulation and determination of the drug distribution profile across the SC

Approximately 20 tapes per application site were cut into pieces of 2.5 x 2.5 cm² and allowed to equilibrate for at least 12 hours before weighing.

600 µl of a gelled formulation was applied using a 1.8 cm diameter Hill Top Chamber® (Hill Top Research, Cincinnati, OH, USA) on the forearm, at least 4 cm from either the wrist or the bend of the elbow. The chamber was fixed to the skin with adhesive tape (Curafix® H, Lohmann & Rauscher, Rengsdorf, Germany). An un-gelled, liquid formulation was applied via a foam tape (3M, St. Paul, MN, USA), into which a 2.0 cm diameter hole had been cut. The foam tape was applied to the forearm (7), a piece of paper towel was inserted and the liquid formulation was added (8), finally, the foam tape system was covered by an occlusive tape (9) to prevent any loss of the formulation.


(7)

(8)

(9)

After the desired application time, the chamber or the foam tape system was removed and the SC at the treated skin site was cleaned with a dry paper towel or by additional twice wiping a 70 % v/v isopropyl alcohol swab (Sterets swab, Seton Healthcare, Oldham, UK). A template (piece of polypropylene foil) into which a 2.0 cm diameter hole had been cut was placed onto the same cleaned, treated skin site and affixed with self-adhesive tape. This template ensured that all tape-strips were removed from the same site. Immediately after, the initial TEWL value was measured. Then, a pre-weighed tape was applied to this template, pressed down 10-times with a constant pressure (140 g/cm²) using a weighted roller and then removed with one quick movement. Up to 20 strips were taken from each treated site. TEWL measurements were performed during the stripping procedure (after every 3-5 tape-strips), which was stopped if TEWL reached 60 g/m²/h, or 4

times the initial TEWL value. This was to ensure, that the SC was never stripped completely but at least 75 % of the SC was removed. Each tape was carefully weighed again after stripping to determine the mass and thickness of SC removed. Afterwards, each tape was inserted into a 2 ml glass vial and the drug was extracted with an appropriate solvent mixture by overnight shaking. The solution obtained was analysed for drug by HPLC.

Data elaboration

The drug concentration in each tape-strip (mg/cm³) was plotted as a function of its position (x) within the normalized SC thickness (L) (Figure 3). These profiles were fitted to the appropriate solution of Fick's 2nd law of diffusion. From the best fit of the experimental data to this Equation, the SC-vehicle partition coefficient and the diffusivity parameter of the drug across the SC was deduced.

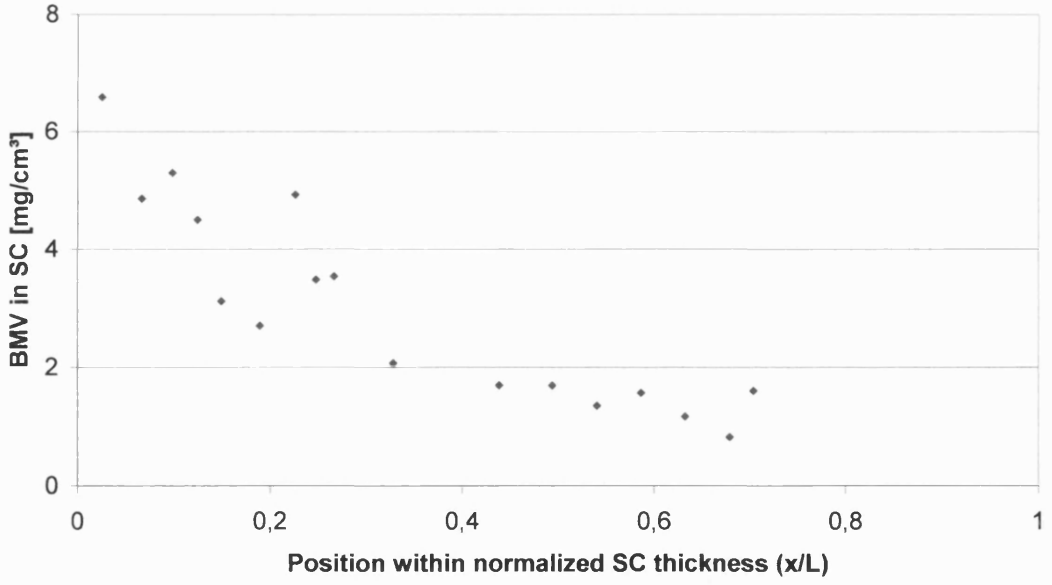


Figure 3: Typical BMV distribution profile across the SC.

3. Vasoconstrictor assay protocol

A maximum of ten application sites were selected on the forearm of a healthy volunteer. The area of each site was approximately 2 cm^2. At least one of these application sites was used for the drug free vehicle; the other ones were treated with drug containing formulations.

The vasoconstrictor assay was carried out according to the "staggered application with synchronized removal" method of the FDA Guidance "Topical Dermatologic Glucocorticoids – *In vivo* Bioequivalence". Before applying the formulations, the application areas on the forearms were marked with a permanent marker. This was to ensure to measure always the same skin sites. The 1.2 cm diameter Hill Top Chambers® (Hill Top Research, Cincinnati, OH, USA) were filled with the formulations, which content approximately 250 µl of volume. Then, the filled chambers were applied to the forearm in a staggered manner and affixed with adhesive tape (Curafix® H, Lohmann & Rauscher, Rengsdorf, Germany). The drug application sites were on the volar forearm, at least 4 cm from the wrist and 4 cm from the antecubital fossa. The amounts of the formulations applied ensured infinite dose conditions.

The formulations were applied to the skin for dose durations of 2, 3, 4, 5 and 6 hours. The drug free vehicles were also applied as control for 6 hours. The chambers were then removed simultaneously and excess formulation was cleaned off with a dry paper towel. The skin blanching response of the treated skin sites (with formulations and with the control) was assessed via a chromameter (CR-300, Minolta, Ahrensburg, Germany) using the a-scale values at 0, 2, 4, 6 19 and 24 hours after formulation removal (10, 11).

(10)

(11)

The chromameter was calibrated using an optically white plate immediately before use. Baseline readings were taken at all sites prior to the application of the formulations. The a-scale readings for each drug application site were adjusted for the baseline value and the control and expressed as the change in this parameter (Δa). As the a-scale values decrease with increasing skin blanching, Δa is negative. The degree of response was therefore expressed as the (positive) area above the response curve (AARC) using the trapezoidal rule.

4. UV spectrophotometer method

The tape-stripping procedure was performed as described above with the following modifications. The template had a size of ~ 5 x 4 cm^2, in which a 2 x 2 cm^2 square had been cut. The tapes for stripping were cut into rectangles of 4.0 x 2.5 cm^2.

After weighing, the same tapes were fixed on a sample holder and the holder with the tape was then transferred into the spectrophotometer, which was equipped with a quadratic beam (1 x 1 cm^2). The absorbance of the SC attached to the tape was quantified at 430 nm (Lambda 35 spectrophotometer, Perkin Elmer, Überlingen, Germany) with an empty tape as reference. The absorbance at 430 nm was taken as a measure because the spectral range around this wavelength is sufficient apart from the absorbance bands of other components of the system. The absorbance of the tape does not disturb the absorbance of the SC. The homogeneous distribution of the SC removed was checked visually immediately after the removal.

5. Data

Table 1: Cumulative SC thickness removed, normalized SC thickness removed and corresponding TEWL values after tape-stripping at an untreated skin site of several volunteers

Volunteer	1			2		
Tape	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL
0	0,00	0,00	8,73	0,00	0,00	8,08
1	0,95	0,06	8,87	0,22	0,02	9,23
2	1,82	0,12	8,78	1,50	0,14	8,89
3	2,47	0,16	8,93	2,05	0,19	9,66
4	3,00	0,20	9,14	2,62	0,24	9,33
5	3,40	0,22	9,30	3,12	0,29	9,66
6	4,03	0,26	9,25	3,42	0,32	10,27
7	4,53	0,30	9,48	3,75	0,35	10,59
8	5,03	0,33	10,05	4,17	0,39	11,01
9	5,43	0,36	11,05	4,52	0,42	11,89
10	6,00	0,39	11,98	4,90	0,46	11,73
11	6,45	0,42	12,74	5,53	0,51	13,86
12	6,73	0,44	13,37	5,80	0,54	14,08
13	7,23	0,47	14,81	6,23	0,58	16,05
14	7,55	0,50	16,16	6,50	0,60	16,88
15	7,93	0,52	16,20	6,80	0,63	19,65
16	8,50	0,56	17,90	7,15	0,67	23,03
17	8,90	0,58	17,99	7,55	0,70	28,26
18	9,43	0,62	18,26	7,75	0,72	36,14
19	9,80	0,64	22,00	7,73	0,72	46,55
20	10,20	0,67	24,29	8,08	0,75	69,25
21	10,95	0,72	26,86			
22	11,53	0,76	36,83			
23	11,95	0,79	50,71			
Total thickness [µm]	15,23			10,75		

Volunteer	3			4		
Tape	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL
0	0,00	0,00	13,11	0,00	0,00	11,01
1	0,43	0,04	13,62	0,60	0,05	11,70
2	0,83	0,07	13,73	1,02	0,09	11,31
3	1,15	0,10	13,04	1,53	0,14	11,57
4	1,95	0,17	13,73	2,07	0,19	12,23
5	2,43	0,21	13,43	2,77	0,25	12,66
6	2,85	0,24	13,54	3,28	0,29	11,93
7	3,35	0,29	15,06	3,76	0,34	13,69
8	3,77	0,32	15,80	4,01	0,36	13,98
9	4,32	0,37	16,43	4,39	0,39	14,34
10	4,82	0,41	16,50	4,87	0,44	16,19
11	5,25	0,45	17,72	5,38	0,48	16,67
12	5,50	0,47	19,25	5,73	0,51	19,82
13	6,10	0,52	22,68	6,18	0,55	20,21
14	6,40	0,55	25,96	6,46	0,58	21,68
15	6,77	0,58	27,68	6,78	0,61	24,54
16	7,22	0,62	35,79	7,10	0,64	27,38
17	7,50	0,64	42,65	7,35	0,66	34,15
18	7,75	0,66	45,53	7,58	0,68	40,88
19	8,05	0,69	60,06			
20						
21						
22						
23						
Total thickness [µm]	11,66			11,86		

Volunteer	5			6		
Tape	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL
0	0,00	0,00	9,50	0,00	0,00	7,63
1	0,86	0,08	8,36	0,48	0,05	7,75
2	1,46	0,13	8,91	0,95	0,09	7,70
3	2,20	0,20	8,90	1,50	0,15	8,19
4	2,64	0,24	9,99	1,95	0,19	8,46
5	3,21	0,29	10,25	2,45	0,24	8,73
6	3,69	0,33	11,27	2,95	0,29	9,37
7	4,11	0,37	12,03	3,35	0,33	9,89
8	5,06	0,45	13,03	3,85	0,38	10,25
9	5,57	0,50	13,89	4,33	0,42	10,95
10	5,98	0,54	15,73	4,73	0,46	11,81
11	6,40	0,57	18,38	5,05	0,49	12,41
12	7,03	0,63	20,49	5,38	0,53	12,94
13	7,42	0,66	23,32	5,88	0,57	15,16
14	7,77	0,70	30,06	6,25	0,61	16,71
15	8,24	0,74	38,23	6,65	0,65	22,98
16	8,69	0,78	49,79	6,98	0,68	29,94
17				7,35	0,72	41,18
18						
19						
20						
21						
22						
23						
Total thickness [µm]	11,16			10,23		

Volunteer	7			8		
Tape	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL
0	0,00	0,00	15,45	0,00	0,00	13,02
1	0,45	0,05	15,48	0,64	0,05	13,84
2	1,02	0,11	16,19	1,21	0,10	14,67
3	1,38	0,14	16,48	2,23	0,18	13,79
4	1,54	0,16	16,11	2,80	0,23	15,00
5	1,90	0,20	17,22	3,15	0,26	16,00
6	2,06	0,21	17,78	3,57	0,29	16,42
7	2,54	0,26	19,73	4,27	0,35	17,92
8	2,83	0,29	20,77	4,49	0,37	18,55
9	2,97	0,31	24,44	4,81	0,39	19,02
10	3,20	0,33	21,92	5,06	0,41	19,72
11	3,47	0,36	27,43	5,35	0,44	21,08
12	3,70	0,39	27,45	6,05	0,50	23,20
13	3,99	0,42	30,74	6,24	0,51	24,64
14	4,17	0,43	32,55	6,40	0,52	24,74
15	4,33	0,45	42,38	6,46	0,53	26,11
16	4,67	0,49	52,89	6,68	0,55	27,32
17				6,97	0,57	29,90
18				7,19	0,59	32,04
19				8,09	0,66	38,50
20				8,47	0,69	44,80
21				9,04	0,74	56,37
22						
23						
Total thickness [µm]	7,17			12,21		

Volunteer	9			10		
Tape	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL
0	0,00	0,00	9,21	0,00	0,00	10,38
1	1,15	0,12	8,59	0,70	0,07	10,14
2	1,53	0,16	9,29	1,31	0,14	10,55
3	2,04	0,21	9,27	1,78	0,19	10,94
4	2,42	0,25	10,77	2,17	0,23	11,06
5	2,83	0,30	10,42	2,85	0,30	11,41
6	3,34	0,35	12,21	3,24	0,34	11,58
7	3,76	0,39	11,96	3,53	0,37	12,65
8	4,07	0,43	12,80	3,86	0,41	13,16
9	4,49	0,47	13,54	4,13	0,43	12,76
10	5,25	0,55	16,24	4,45	0,47	13,80
11	5,73	0,60	17,81	4,74	0,50	14,76
12	5,89	0,62	21,68	5,04	0,53	15,67
13	6,24	0,66	23,88	5,37	0,56	16,64
14	6,40	0,67	28,16	5,60	0,59	17,72
15	6,65	0,70	39,10	5,82	0,61	19,78
16				5,99	0,63	21,90
17				6,23	0,65	23,98
18				7,19	0,76	28,73
19				7,59	0,80	31,33
20				8,13	0,86	34,35
21				8,42	0,89	37,71
22						
23						
Total thickness [µm]	9,51			11,32		

Volunteer	11		
Tape	Cumulative SC thickness [µm]	Normalized SC thickness	TEWL
0	0,00	0,00	12,15
1	0,45	0,05	12,37
2	0,72	0,08	12,65
3	0,98	0,10	12,84
4	1,35	0,14	14,10
5	1,68	0,18	13,82
6	1,98	0,21	14,76
7	2,60	0,27	16,65
8	2,80	0,29	16,95
9	3,42	0,36	17,67
10	3,75	0,39	18,40
11	4,05	0,42	19,62
12	4,32	0,45	22,63
13	4,52	0,47	22,20
14	4,80	0,50	24,08
15	5,05	0,53	23,93
16	5,65	0,59	29,31
17	6,00	0,63	30,75
18	6,50	0,68	34,38
19	6,82	0,71	42,17
20	7,20	0,75	49,13
21	7,27	0,76	57,68
22			
23			
Total thickness [µm]	9,55		

Table 2: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80 % of saturation in MCT. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,080	1,463	0,054	1,150	0,069	1,767
0,196	1,043	0,144	0,545	0,202	1,494
0,253	0,906	0,207	0,816	0,302	1,223
0,297	0,833	0,256	0,632	0,363	1,151
0,335	0,675	0,299	0,613	0,410	0,938
0,370	0,954	0,338	0,313	0,451	0,689
0,407	0,726	0,374	0,315	0,488	0,607
0,430	0,733	0,423	0,503	0,567	0,467
0,450	0,628	0,474	0,423	0,667	0,365
0,481	0,443	0,581	0,145	0,727	0,347
0,540	0,436	0,637	0,250		
0,572	0,250	0,664	0,245		
0,595	0,390	0,692	0,275		
0,615	0,260	0,719	0,165		
0,641	0,185				
0,675	0,236				
0,708	0,280				
0,732	0,210				
0,761	0,183				
0,796	0,168				
0,829	0,075				
0,908	0,130				
0,950	0,182				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,087	1,379	0,071	1,363	0,132	0,702
0,176	0,990	0,180	0,745	0,318	0,421
0,248	0,911	0,243	0,557	0,422	0,300
0,311	0,644	0,290	0,591	0,516	0,211
0,372	0,544	0,325	0,463	0,601	0,137
0,430	0,685	0,351	0,617	0,679	0,088
0,483	0,792	0,368	0,630	0,756	0,079
0,521	0,521	0,380	0,390	0,863	0,057
0,549	0,484	0,396	0,394	0,957	0,067
0,577	0,320	0,408	0,975		
0,602	0,186	0,422	0,540		
0,631	0,473	0,464	0,542		
0,658	0,437	0,508	0,255		
0,676	0,314	0,533	0,135		
		0,560	0,113		
		0,600	0,086		
		0,640	0,150		
		0,689	0,064		
		0,766	0,218		
		0,794	0,242		
		0,820	0,156		

Table 3: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80 % of saturation in ME. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,025	6,585	0,036	8,249	0,041	11,290
0,067	4,855	0,106	6,302	0,135	3,860
0,098	5,297	0,167	5,067	0,232	5,260
0,124	4,500	0,281	2,882	0,315	4,150
0,149	3,115	0,401	3,413	0,397	4,060
0,189	2,700	0,461	2,703	0,459	3,630
0,226	4,927	0,511	1,915	0,501	3,220
0,247	3,485	0,576	1,615	0,545	2,140
0,266	3,540	0,632	2,124	0,587	1,639
0,328	2,072	0,659	2,080	0,618	2,260
0,439	1,698	0,683	1,556	0,640	2,143
0,494	1,696	0,705	1,983	0,682	2,163
0,540	1,350	0,721	2,022	0,721	1,860
0,586	1,568	0,733	2,029	0,740	1,791
0,632	1,168	0,752	1,600	0,760	1,717
0,679	0,821				
0,704	1,600				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,085	7,293	0,028	7,487	0,049	7,203
0,169	3,678	0,081	3,735	0,124	5,914
0,271	2,606	0,180	2,511	0,218	3,869
0,362	2,293	0,304	1,570	0,522	2,228
0,451	1,273	0,390	0,864	0,605	1,363
0,521	1,459	0,438	1,350	0,662	1,372
0,569	1,453	0,460	1,125	0,712	1,168
0,601	1,843	0,485	1,015	0,759	1,344
0,627	1,153	0,513	1,050		
0,666	1,348	0,538	0,600		
		0,555	1,688		
		0,571	0,428		

Table 4: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80 % of saturation in TCL. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,073	93,110	0,053	69,257	0,085	103,230
0,190	67,638	0,138	47,096	0,234	98,027
0,290	51,266	0,200	34,508	0,349	83,316
0,383	30,789	0,257	20,348	0,438	73,195
0,448	31,746	0,303	22,400	0,580	57,633
0,499	13,078	0,337	18,783	0,617	44,067
0,541	10,500	0,381	16,738	0,643	30,873
0,575	8,013	0,414	16,600	0,666	21,791
0,609	6,525	0,485	16,982	0,684	30,500
0,638	6,725	0,505	19,300	0,700	27,844
0,660	10,900	0,521	17,025	0,715	22,050
0,681	9,736	0,543	19,509	0,733	11,673
0,704	5,855			0,753	16,233
0,726	5,530			0,768	14,483
0,745	7,763				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,061	80,532	0,053	101,556	0,061	100,263
0,172	45,300	0,131	95,794	0,171	68,274
0,266	35,948	0,190	58,418	0,264	38,329
0,340	29,457	0,254	58,247	0,344	43,704
0,418	21,770	0,310	22,365	0,409	28,843
0,492	14,133	0,353	18,127	0,461	37,547
0,547	8,647	0,388	20,145	0,509	16,253
0,594	8,080	0,420	14,390	0,563	17,805
0,631	13,475	0,453	6,791	0,636	23,094
0,730	3,085	0,484	6,922	0,705	11,269
0,872	2,893	0,526	12,661	0,742	16,280
		0,562	17,480	0,770	6,840
		0,587	5,655	0,803	4,815
		0,616	5,188	0,836	5,280
				0,870	4,536

Table 5: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 10 % of saturation in MCT. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,045	0,259	0,019	0,250	0,061	0,131
0,112	0,146	0,101	0,091	0,147	0,132
0,149	0,067	0,128	0,095	0,200	0,041
0,176	0,057	0,210	0,157	0,263	0,044
0,197	0,067	0,297	0,088	0,401	0,040
0,219	0,229	0,353	0,123	0,468	0,030
0,238	0,100	0,401	0,051	0,546	0,050
0,259	0,044	0,441	0,031		
0,285	0,000	0,483	0,092		
0,308	0,000	0,513	0,055		
0,356	0,052	0,537	0,100		
0,415	0,071				
0,455	0,060				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,050	0,083	0,069	0,131	0,047	0,086
0,107	0,300	0,167	0,070	0,116	0,033
0,142	0,146	0,212	0,039	0,154	0,098
0,245	0,020	0,238	0,069	0,184	0,031
0,377	0,038	0,257	0,206	0,367	0,010
0,502	0,012	0,286	0,036	0,535	0,014
0,623	0,018	0,347	0,060	0,573	0,032
0,707	0,027	0,369	0,036	0,598	0,000
0,757	0,027	0,390	0,070	0,622	0,000
0,788	0,046	0,426	0,033	0,643	0,017
		0,467	0,036		
		0,506	0,020		
		0,552	0,041		

Table 6: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 10 % of saturation in ME. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,044	0,808	0,041	0,708	0,056	0,734
0,107	0,775	0,101	0,302	0,139	0,428
0,142	0,444	0,142	0,233	0,203	0,261
0,174	0,580	0,197	0,220	0,318	0,192
0,206	0,256	0,306	0,150	0,448	0,114
0,228	0,775	0,405	0,131	0,515	0,284
0,361	0,126	0,481	0,123	0,561	0,059
0,542	0,141	0,554	0,176	0,631	0,146
0,611	0,189				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,033	0,480	0,069	0,790	0,040	0,572
0,155	0,199	0,174	0,611	0,129	0,216
0,308	0,111	0,242	0,370	0,235	0,188
0,493	0,052	0,337	0,307	0,339	0,130
0,675	0,061	0,450	0,222	0,424	0,042
0,765	0,066	0,518	0,290	0,479	0,080
0,820	0,020	0,572	0,132	0,519	0,030
0,863	0,000	0,703	0,084		
		0,837	0,243		
		0,887	0,155		
		0,919	0,063		

Table 7: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 1.3 % of saturation in ME. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,045	0,159	0,031	0,056	0,045	0,076
0,109	0,164	0,076	0,051	0,119	0,041
0,147	0,142	0,100	0,040	0,193	0,046
0,221	0,050	0,131	0,038	0,371	0,033
0,392	0,041	0,184	0,014	0,430	0,038
0,542	0,000	0,241	0,030	0,542	0,016
0,601	0,000	0,334	0,025	0,656	0,022
0,661	0,095				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,058	0,069	0,045	0,055	0,038	0,068
0,168	0,026	0,123	0,064	0,097	0,089
0,310	0,044	0,207	0,034	0,186	0,031
0,465	0,055	0,290	0,017	0,309	0,040
0,577	0,009	0,349	0,013	0,425	0,018
0,642	0,000	0,392	0,000	0,496	0,000
0,682	0,000	0,445	0,038	0,521	0,000
0,713	0,000	0,531	0,023		
		0,647	0,014		

Table 8: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80% of saturation in MCT. Skin surface was cleaned using an isopropyl alcohol swab post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,093	0,791	0,052	0,791	0,068	1,172
0,231	0,526	0,130	0,506	0,173	0,533
0,314	0,489	0,182	0,453	0,239	0,500
0,373	0,510	0,222	0,310	0,292	0,328
0,414	0,328	0,256	0,185	0,340	0,235
0,448	0,293	0,289	0,300	0,386	0,265
0,477	0,300	0,315	0,378	0,442	0,158
0,510	0,300	0,341	0,525	0,500	0,144
0,541	0,243	0,363	0,286	0,546	0,350
0,588	0,260	0,404	0,327	0,587	0,236
0,603	0,340			0,685	0,159

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,025	0,353	0,082	0,349	0,037	0,261
0,088	0,174	0,233	0,191	0,110	0,159
0,218	0,089	0,349	0,175	0,189	0,103
0,360	0,100	0,440	0,115	0,341	0,112
0,458	0,059	0,530	0,091	0,506	0,090
0,550	0,038	0,610	0,100	0,687	0,038
0,637	0,038	0,671	0,105	0,865	0,050
0,762	0,037	0,712	0,050	0,939	0,060
		0,742	0,000	0,990	0,028

Table 9: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80% of saturation in ME. Skin surface was cleaned using an isopropyl alcohol swab post-application of gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,069	1,991	0,024	3,688	0,033	3,365
0,180	0,788	0,068	1,064	0,086	1,792
0,261	0,329	0,101	2,588	0,147	1,200
0,360	0,202	0,127	1,470	0,254	0,705
0,464	0,091	0,166	1,694	0,425	0,548
0,537	0,108	0,216	1,322	0,603	0,348
0,576	0,255	0,289	0,981	0,722	0,175
0,639	0,151	0,372	0,920	0,788	0,600
0,703	0,200	0,443	0,578		
0,738	0,463	0,492	0,990		

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,075	1,943	0,058	1,205	0,046	1,577
0,262	1,086	0,212	0,616	0,150	1,076
0,444	0,580	0,371	0,370	0,252	0,679
0,581	0,422	0,509	0,284	0,361	0,437
0,703	0,169	0,629	0,143	0,484	0,251
0,807	0,148	0,718	0,131	0,559	0,347
0,913	0,149	0,776	0,313	0,649	0,104
		0,796	0,360	0,781	0,117
				0,856	0,255
				0,895	0,111
				0,929	0,143

Table 10: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80% of saturation in MCT. Skin surface was cleaned using a dry paper towel post-application of un-gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,047	1,227	0,022	1,025	0,049	0,939
0,128	0,709	0,055	0,567	0,115	0,733
0,185	0,409	0,075	0,400	0,162	0,517
0,230	0,365	0,095	0,233	0,220	0,358
0,274	0,423	0,120	0,213	0,265	0,279
0,316	0,211	0,138	0,400	0,299	0,291
0,349	0,107	0,151	0,200	0,343	0,252
0,374	0,590	0,171	0,150	0,386	0,155
0,389	0,825	0,195	0,129	0,420	0,264
0,398	0,300	0,213	0,300	0,456	0,108
0,414	0,510	0,238	0,245	0,482	0,229
0,444	0,242	0,273	0,150	0,506	0,145
0,475	0,250	0,298	0,117	0,532	0,325
0,489	0,325	0,349	0,414	0,556	0,056
0,508	0,443	0,404	0,213		
0,563	0,124	0,447	0,138		
0,627	0,096				
0,667	0,057				
0,695	0,162				
0,737	0,044				

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,027	0,576	0,044	0,361	0,054	0,648
0,118	0,220	0,115	0,161	0,135	0,381
0,243	0,111	0,163	0,115	0,181	0,264
0,347	0,081	0,202	0,092	0,235	0,200
0,561	0,037	0,266	0,118	0,290	0,150
0,784	0,047	0,313	0,084	0,340	0,112
0,901	0,041	0,389	0,121	0,492	0,038
0,982	0,150	0,423	0,200	0,707	0,060
		0,459	0,073	0,000	

Table 11: BMV concentrations [mg/cm³] across the SC (x/L) following a 2-hour application of the drug at 80% of saturation in ME. Skin surface was cleaned using a dry paper towel post-application of un-gelled vehicles.

Volunteer 1		Volunteer 2		Volunteer 3	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,035	6,888	0,021	6,229	0,045	4,636
0,085	6,167	0,052	3,586	0,126	2,792
0,118	3,382	0,071	3,300	0,248	1,858
0,164	2,567	0,104	1,431	0,412	0,991
0,219	2,016	0,178	1,250	0,542	0,523
0,265	1,068	0,261	1,527	0,676	0,471
0,294	1,556	0,339	1,687	0,771	1,573
0,341	1,036	0,429	1,443	0,819	0,479
0,409	0,655	0,487	1,333		
0,460	2,005	0,529	1,037		
0,490	3,400	0,563	2,475		

Volunteer 4		Volunteer 5		Volunteer 6	
x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]	x/L	Cx [mg/cm³]
0,030	4,644	0,117	1,033	0,036	3,464
0,105	2,159	0,339	0,475	0,093	2,553
0,217	1,120	0,513	0,250	0,145	1,552
0,338	0,858	0,632	0,119	0,216	1,147
0,442	0,503	0,744	0,075	0,291	0,893
0,532	0,604	0,846	0,092	0,361	0,681
0,647	0,636	0,910	0,033	0,555	0,520
0,763	0,615			0,743	0,750
0,842	0,929				

Table 12: DPK parameter deduced from the BMV distribution profiles following a 2-hour application. BMV concentration was adjusted to different levels of saturation in several vehicles. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

	K					
Volunteer	MCT 2h 80%	ME 2h 80%	TCL 2h 80%	MCT 2h 10%	ME 2h 10%	ME 2h 1.3%
1	0,96	0,69	1,12	1,24	0,73	1,27
2	0,62	0,79	0,67	0,86	0,57	0,38
3	1,22	0,96	1,29	0,69	0,58	0,45
4	0,83	0,77	0,86	0,98	0,44	0,45
5	0,80	0,68	1,28	0,66	0,68	0,43
6	0,54	0,78	1,02	0,40	0,49	0,59

	D/L^2 [1/h]					
Volunteer	MCT 2h 80%	ME 2h 80%	TCL 2h 80%	MCT 2h 10%	ME 2h 10%	ME 2h 1.3%
1	0,058	0,076	0,032	0,012	0,032	0,026
2	0,059	0,075	0,033	0,068	0,010	0,025
3	0,066	0,066	0,097	0,032	0,031	0,082
4	0,080	0,039	0,030	0,025	0,012	0,057
5	0,045	0,023	0,019	0,048	0,052	0,037
6	0,046	0,060	0,044	0,031	0,013	0,022

	AUC [mg/cm^3]					
Volunteer	MCT 2h 80%	ME 2h 80%	TCL 2h 80%	MCT 2h 10%	ME 2h 10%	ME 2h 1.3%
1	0,49	2,65	32,17	0,05	0,25	0,05
2	0,39	3,03	19,43	0,07	0,11	0,01
3	0,81	3,48	57,27	0,04	0,19	0,03
4	0,59	2,23	23,98	0,05	0,09	0,02
5	0,45	1,54	28,21	0,05	0,29	0,02
6	0,31	2,75	33,99	0,02	0,11	0,02

	Cs,sc [mg/ml]					
Volunteer	ME 2h 80%	ME 2h 10%	ME 2h 1.3%	MCT 2h 80%	MCT 2h 10%	TCL 2h 80%
1	8,12	8,54	14,84	1,70	2,59	141,25
2	9,29	6,70	4,43	1,30	1,81	84,16
3	11,19	6,80	5,22	2,56	1,44	162,04
4	9,01	5,10	5,25	1,74	2,06	108,16
5	7,97	7,97	4,98	1,67	1,38	161,78
6	9,18	5,71	6,96	1,14	0,85	129,02

Table 13: DPK parameter deduced from the BMV distribution profiles following a 6-hour application. BMV concentration was adjusted to 80% of the saturation level in MCT and ME. Skin surface was cleaned using a dry paper towel post-application of gelled vehicles.

	MCT 6h 80%			
Volunteer	K	D/L² [1/h]	Cs,sc [mg/ml]	AUC [mg/cm³]
1	0,88	0,024	1,85	0,60
2	0,48	0,044	1,00	0,38
3	0,77	0,044	1,62	0,61
4	0,92	0,025	1,93	0,63
5	0,93	0,055	1,96	0,77
6	0,65	0,029	1,37	0,47

	ME 6h 80%			
Volunteer	K	D/L² [1/h]	Cs,sc [mg/ml]	AUC [mg/cm³]
1	0,69	0,035	8,04	2,86
2	0,85	0,026	9,93	3,27
3	0,83	0,032	9,66	3,38
4	1,29	0,043	15,03	5,59
5	1,11	0,023	12,95	4,09
6	1,30	0,056	15,22	5,87

Table 14: DPK parameter deduced from the BMV distribution profiles following a 2-hour application. BMV concentration was adjusted to 80% of the saturation level in MCT and ME. Skin surface was cleaned using an isopropyl alcohol swab post-application of gelled vehicles.

	MCT 2h 80%			
Volunteer	K	D/L² [1/h]	Cs,sc [mg/ml]	AUC [mg/cm³]
1	0,51	0,074	1,07	0,35
2	0,42	0,046	0,89	0,24
3	0,73	0,023	1,53	0,30
4	0,18	0,022	0,38	0,07
5	0,22	0,055	0,46	0,14
6	0,13	0,073	0,27	0,09

	ME 2h 80%			
Volunteer	K	D/L² [1/h]	Cs,sc [mg/ml]	AUC [mg/cm³]
1	0,31	0,007	3,63	0,39
2	0,29	0,031	3,42	0,76
3	0,40	0,006	4,68	0,47
4	0,24	0,042	2,80	0,72
5	0,13	0,051	1,50	0,42
6	0,18	0,031	2,09	0,47

Table 15: DPK parameter deduced from the BMV distribution profiles subsequent to a 2-hour application. BMV concentration was adjusted to 80% of the saturation level in MCT and ME. Skin surface was cleaned using a dry paper towel post-application of un-gelled (liquid) vehicles.

	MCT 2h 80%			
Volunteer	K	D/L^2 [1/h]	Cs,sc [mg/ml]	AUC [mg/cm^3]
1	0,58	0,046	1,22	0,33
2	0,46	0,008	0,96	0,11
3	0,57	0,025	1,19	0,24
4	0,38	0,006	0,79	0,08
5	0,15	0,045	0,32	0,09
6	0,46	0,011	0,97	0,13

	ME 2h 80%			
Volunteer	K	D/L^2 [1/h]	Cs,sc [mg/ml]	AUC [mg/cm^3]
1	0,77	0,014	9,03	1,34
2	0,42	0,051	4,88	1,37
3	0,48	0,033	5,66	1,30
4	0,52	0,009	6,13	0,73
5	0,14	0,036	1,70	0,41
6	0,40	0,013	4,65	0,68

Table 16: Amount of BMV in the SC as a function of time after application of BMV in MCT at 80% of the saturation level. For the uptake phase, formulations were removed at 2, 4 and 6 hours and subsequently tape-stripped. For the elimination phase, formulations were maintained to the skin for 6 hours, subsequently removed, and the SC was stripped after a further 2, 6 and 24 hours.

	Amount of BMV in the SC [$\mu g/cm^2$]					
time [h]	Volunteer1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6
0	0	0	0	0	0	0
2	0,83	0,61	0,68	0,57	0,90	0,74
4	0,91	0,66	0,85	0,67	1,34	0,72
6	1,70	0,80	1,02	1,07	1,33	0,98
8	1,10	0,57	0,84	0,65	0,92	0,54
12	1,00	0,46	0,69	0,61	0,75	0,50
30	0,28	0,41	0,58	0,29	0,24	0,34
AUC [$\mu g*h/cm^2$]	22,87	13,99	19,75	15,32	19,41	14,32

Table 17: Amount of BMV in the SC as a function of time after application of BMV in ME at 80% of the saturation level. For the uptake phase, formulations were removed at 2, 4 and 6 hours and subsequently tape-stripped. For the elimination phase, formulations were maintained to the skin for 6 hours, subsequently removed, and the SC was stripped after a further 2, 6 and 24 hours.

	Amount of BMV in the SC [µg/cm²]					
time [h]	Volunteer1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6
0	0	0	0	0	0	0
2	7,10	4,27	5,44	7,99	7,16	4,22
4	5,21	4,89	6,11	7,61	5,19	9,65
6	7,94	8,08	6,68	7,23	4,71	4,38
8	3,51	3,56	4,16	4,81	3,90	3,54
12	3,15	2,76	1,91	4,41	3,41	1,81
30	2,21	1,04	0,96	1,04	1,01	0,94
AUC [µg*h/cm²]	98,47	80,61	73,15	109,97	85,26	71,27

Table 18a: Chromameter a-scale raw data for a volunteer following a 4-hour dose duration of BMV at different concentrations in ME. BL = baseline.

Measurement time [h]	Control	1,2 mg/ml	0,6 mg/ml	0,3 mg/ml	0,15 mg/ml	0,07 mg/ml	0,04 mg/ml
BL (-5)	7,43	8,32	7,01	8,45	6,39	7,52	6,97
0	6,60	6,77	5,28	7,46	6,00	6,97	6,16
2	7,70	6,27	5,07	6,70	5,68	6,70	6,42
4	7,87	6,49	5,06	6,52	5,59	6,54	6,79
6	7,78	5,96	5,27	6,53	5,78	6,69	6,67
19	7,05	6,62	6,20	7,13	6,05	7,09	6,86
24	7,85	8,17	6,95	7,48	6,34	7,49	7,06

Table 18b: Baseline-adjusted a-scale data for a volunteer following a 4-hour dose duration of BMV at different concentrations in ME.

Measurement time [h]	Control	1,2 mg/ml	0,6 mg/ml	0,3 mg/ml	0,15 mg/ml	0,07 mg/ml	0,04 mg/ml
BL (-5)	0	0	0	0	0	0	0
0	-0,83	-1,55	-1,73	-0,99	-0,39	-0,55	-0,81
2	0,27	-2,05	-1,94	-1,75	-0,71	-0,82	-0,55
4	0,44	-1,83	-1,95	-1,93	-0,80	-0,98	-0,18
6	0,35	-2,36	-1,74	-1,92	-0,61	-0,83	-0,30
19	-0,38	-1,70	-0,81	-1,32	-0,34	-0,43	-0,11
24	0,42	-0,15	-0,06	-0,97	-0,05	-0,03	0,09

Table 18c: Baseline-adjusted, control site-corrected a-scale data and AARC(0-24) for a volunteer following 4-hour dose duration of BMV at different concentrations in ME.

Measurement time [h]	Control	1,2 mg/ml	0,6 mg/ml	0,3 mg/ml	0,15 mg/ml	0,07 mg/ml	0,04 mg/ml
BL (-5)	0	0	0	0	0	0	0
0	0	-0,72	-0,90	-0,16	0,44	0,28	0,02
2	0	-2,32	-2,21	-2,02	-0,98	-1,09	-0,82
4	0	-2,27	-2,39	-2,37	-1,24	-1,42	-0,62
6	0	-2,71	-2,09	-2,27	-0,96	-1,18	-0,65
19	0	-1,32	-0,43	-0,94	0,04	-0,05	0,27
24	0	-0,57	-0,48	-1,39	-0,47	-0,45	-0,33
AARC(0-24)		-43,53	-30,85	-37,90	-12,12	-15,22	-6,06

Table 19: Baseline-adjusted, control site-corrected a-scale data following different dose durations of BMV at 80% of the saturation level in MCT.

DD = 2h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-0,50	-1,30	-1,82	-1,94	-0,98	-0,77
2	-0,54	-1,26	-1,25	-1,37	-0,90	-0,50
3	-0,52	-0,58	-2,28	-3,05	-1,96	-0,60
4	-0,75	-1,23	-1,62	-2,50	-1,05	-1,06
5	-0,40	-1,15	-2,81	-2,56	-0,40	-0,53
6	-0,55	-2,54	-2,90	-2,60	-1,25	-0,53

DD = 3h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,00	-1,72	-1,48	-2,50	-0,97	0,00
2	-2,89	-3,13	-3,38	-3,14	-0,40	0,00
3	-0,28	-0,24	-2,48	-2,15	-1,24	-1,06
4	0,00	0,27	-1,00	-2,25	-1,18	-0,81
5	-1,04	-1,77	-2,83	-2,94	-0,87	-0,75
6	-1,04	-2,28	-3,03	-3,01	-1,33	-0,60

DD = 4h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-1,40	-2,89	-2,23	-2,82	-0,80	-0,19
2	-2,33	-3,49	-3,75	-3,77	-1,35	-0,33
3	-1,59	-1,18	-2,06	-3,03	-1,38	-1,69
4	-1,63	-0,65	-1,99	-2,99	-1,67	-0,70
5	-1,43	-2,17	-2,88	-3,05	-0,57	-0,48
6	-1,20	-1,15	-1,59	-1,89	-0,86	-0,46

DD = 5h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-1,76	-3,72	-2,56	-3,51	-1,79	-0,82
2	-1,89	-1,94	-2,70	-2,97	-0,15	-0,05
3	-1,66	-1,42	-3,24	-2,80	-1,30	-0,56
4	-1,69	-2,30	-2,84	-3,32	-1,13	-0,69
5	-1,85	-2,45	-2,05	-2,66	-0,68	-0,14
6	-0,56	-2,27	-2,28	-2,96	-1,07	-1,00

DD = 6h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-2,54	-2,33	-3,09	-3,59	-1,28	-0,37
2	-1,98	-2,14	-2,89	-3,26	-0,36	-0,55
3	-1,51	-0,98	-1,87	-2,18	-1,07	-0,17
4	-1,76	-2,83	-2,77	-3,37	-2,19	-1,00
5	-1,55	-2,41	-2,57	-2,96	-0,61	-0,55
6	-3,00	-3,21	-3,15	-3,51	-1,29	-0,38

Table 20: Baseline-adjusted, control site-corrected a-scale data following different dose durations of BMV at 80% of the saturation level in ME.

DD = 2h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-1,45	-1,72	-3,83	-3,70	-1,68	-0,76
2	-1,27	-1,41	-2,15	-2,57	-0,44	-0,12
3	-1,02	-1,10	-1,85	-2,40	-1,67	-1,07
4	-0,22	-1,46	-2,06	-3,49	-1,56	-0,14
5	-0,82	-1,75	-2,76	-3,81	-1,17	-0,21
6	-0,80	-1,94	-1,43	-2,37	-1,08	-0,63

DD = 3h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,00	-0,42	-2,80	-2,61	-1,15	0,00
2	-0,60	-2,48	-2,67	-3,65	-0,93	-0,75
3	0,00	-1,88	-1,37	-2,14	-0,59	-0,60
4	0,00	-1,54	-2,14	-3,51	-1,61	-0,59
5	-1,12	-2,72	-3,67	-3,55	-1,43	-1,06
6	0,00	-1,25	-1,67	-2,19	-0,85	-0,56

DD = 4h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-0,52	-2,22	-3,08	-3,48	-1,72	-0,12
2	-2,05	-3,13	-3,11	-3,22	-0,68	-0,70
3	-0,30	-2,16	-1,82	-2,91	-1,29	-0,77
4	-0,90	-1,51	-1,79	-2,18	-0,57	-0,40
5	-1,93	-2,33	-3,02	-3,40	-1,17	-0,84
6	-2,03	-1,77	-2,87	-3,35	-1,33	-0,47

DD = 5h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-2,22	-1,90	-3,47	-3,99	-0,54	0,00
2	-2,62	-3,28	-3,17	-3,26	-0,80	-0,89
3	-1,40	-3,40	-2,51	-2,44	-2,10	-0,31
4	-0,21	-1,87	-2,65	-3,60	-1,38	0,00
5	-2,68	-2,76	-3,08	-3,15	-0,82	-0,67
6	-1,58	-2,01	-2,68	-3,22	-1,02	-0,35

DD = 6h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-2,15	-2,56	-2,86	-3,06	-0,97	-0,40
2	-2,91	-3,02	-2,97	-3,13	-1,00	-0,37
3	-1,31	-3,13	-3,00	-3,58	-1,72	-0,75
4	-2,37	-3,09	-3,62	-4,39	-2,11	-1,11
5	-2,04	-1,95	-3,04	-3,58	-0,17	-0,23
6	-2,66	-2,11	-2,90	-3,69	-0,98	-0,98

Table 21: Baseline-adjusted, control site-corrected a-scale data following different dose durations of BMV at 80% of the saturation level in TCL.

DD = 2h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,00	0,00	-0,48	-3,44	-0,81	0,00
2	0,00	-1,40	-1,42	-1,27	0,00	0,00
3	-0,50	-2,02	-1,61	-1,47	0,00	0,00
4	-0,28	-1,58	-2,85	-1,49	-0,39	-0,17
5	0,00	-1,25	-1,81	-2,90	-0,47	-0,57
6	0,00	-1,82	-1,68	-2,07	-1,18	-0,06

DD = 3h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,00	-0,29	-1,39	-3,50	-0,62	0,00
2	-1,15	-1,15	-2,24	-1,57	0,00	0,00
3	-1,04	-2,80	-2,15	-1,96	-0,48	0,00
4	-0,10	-1,41	-2,28	-1,51	-0,82	-0,51
5	-0,68	-0,51	-1,41	-1,87	0,00	0,00
6	-0,29	-2,46	-2,38	-2,24	-1,30	-0,90

DD = 4h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-0,62	-1,88	-1,72	-3,75	-1,66	-0,10
2	-1,39	-0,74	-1,47	-1,34	-0,40	-0,20
3	-1,61	-3,11	-2,08	-2,25	-1,60	-0,20
4	-1,41	-1,78	-3,65	-2,10	-0,50	-0,10
5	-0,98	-0,92	-1,12	-2,21	-0,19	-0,50
6	-0,71	-2,40	-1,85	-1,78	-0,39	-0,10

DD = 5h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-1,86	-2,27	-3,56	-3,85	-1,39	0,00
2	-2,90	-2,77	-2,38	-2,76	0,00	0,00
3	-2,84	-3,30	-2,78	-2,49	-0,26	-0,20
4	-2,13	-1,76	-3,20	-2,71	-0,69	-0,72
5	-0,88	-1,92	-1,79	-2,34	0,00	0,00
6	-1,07	-2,59	-2,01	-2,60	-0,25	-0,48

DD = 6h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-1,70	-2,23	-3,67	-3,70	-0,65	0,00
2	-2,94	-2,82	-2,91	-2,79	0,00	0,00
3	-3,10	-3,58	-3,50	-3,33	-0,87	-0,53
4	-0,20	-2,45	-2,51	-3,12	-0,15	-0,30
5	-1,58	-1,74	-2,18	-3,34	-0,44	-0,25
6	-1,17	-2,47	-1,60	-2,52	-0,34	0,00

Table 22: Baseline-adjusted, control site-corrected a-scale data following different dose durations of BMV at 80% of the saturation level in LMO.

DD = 2h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,78	0,62	0,67	-0,08	0,37	-1,09
2	0,85	-1,19	-2,41	-0,15	-0,12	-0,58
3	0,90	0,63	-1,71	-0,89	0,38	-0,10
4	0,05	0,18	-0,09	-0,74	-1,20	-0,17
5	0,00	0,73	-0,10	-0,02	-1,85	-0,52
6	0,84	-0,05	-0,08	-0,25	-0,61	-0,69

DD = 3h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,00	0,00	-0,31	-1,02	0,00	-0,88
2	0,00	-2,01	-2,13	-1,30	0,00	-0,92
3	-0,49	-0,38	-2,68	-2,75	-1,34	-0,76
4	-0,57	0,00	-0,16	-0,06	-1,04	-0,47
5	0,00	-0,24	-1,56	-1,91	-2,00	-0,31
6	-0,98	-0,49	-1,22	-0,60	-0,19	-0,44

DD = 4h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,82	0,44	-0,49	-0,06	-1,74	-0,66
2	1,10	-1,33	-0,90	-1,44	-0,98	-0,65
3	-1,69	-0,54	-1,09	-1,07	-1,19	-1,18
4	1,36	-0,31	-1,18	-1,27	-0,60	-0,64
5	-0,45	0,15	-0,97	-1,44	-0,71	-0,63
6	0,26	-0,68	-1,69	-1,86	-2,06	-1,20

DD = 5h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	0,00	0,00	-0,22	-0,80	0,09	0,10
2	0,00	-1,11	-1,51	-1,56	-0,68	-0,28
3	-0,76	-0,89	-1,22	-1,56	-0,62	-1,77
4	-0,66	-0,65	-0,94	-2,30	-0,78	-0,35
5	-1,19	-0,71	-1,46	-1,46	-0,86	-0,88
6	-0,74	-2,14	-2,15	-2,00	0,02	-0,69

DD = 6h

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-0,75	-1,34	-1,76	-2,15	-1,25	-1,37
2	0,00	-1,03	-2,98	-1,12	-0,56	-0,09
3	-0,56	-1,57	-1,76	-3,02	-0,48	-1,05
4	-0,80	-0,88	-1,61	-1,69	-0,85	-1,39
5	-0,03	-0,41	-0,89	-0,82	-1,60	-0,97
6	-2,39	-2,54	-2,09	-1,83	-0,15	-0,62

Table 23: Baseline-adjusted, control site-corrected a-scale data following a 4-hour dose durations of BMV at different levels of the saturation level in ME.

10,43%

Time [h]	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8
0	-1,17	-1,60	-0,52	-0,72	-1,35	-2,23	0,00	-0,82
2	-1,40	-1,94	-2,26	-2,32	-3,02	-2,91	-1,54	-1,84
4	-2,31	-2,98	-2,93	-2,27	-2,92	-2,60	-2,99	-2,18
6	-2,66	-3,28	-3,24	-2,71	-3,41	-2,87	-3,74	-2,28
19	-2,47	-1,32	-1,60	-1,32	-1,12	-0,61	-1,17	-0,58
24	-0,56	-0,68	-0,15	-0,57	0,00	-0,19	-0,36	-0,63

5,21%

Time [h]	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8
0	-0,63	-0,30	-0,03	-0,90	-1,63	-1,29	0,00	-0,57
2	-1,67	-1,64	-0,91	-2,21	-3,44	-2,57	-1,25	-1,75
4	-1,82	-2,49	-1,46	-2,39	-3,15	-2,73	-3,49	-2,07
6	-2,56	-2,22	-2,83	-2,09	-3,32	-2,80	-3,67	-2,73
19	-0,42	-0,96	-0,95	-0,43	-0,18	-0,91	-0,90	-0,54
24	-1,14	-0,40	-0,48	-0,48	-0,10	-0,35	-0,61	-0,68

2,61%

Time [h]	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8
0	0,00	0,00	-0,07	-0,16	-0,81	-1,02	0,00	0,00
2	-0,02	-0,32	-0,70	-2,02	-2,82	-2,66	-0,49	-0,96
4	-0,13	-2,00	-1,91	-2,37	-3,44	-2,43	-2,30	-1,26
6	-1,58	-1,97	-2,20	-2,27	-3,07	-2,01	-2,49	-1,39
19	-0,19	-0,32	-0,57	-0,94	-1,88	-0,69	-0,33	-0,25
24	-0,64	0,00	0,00	-1,39	-0,67	-0,33	0,00	0,00

1,30%

Time [h]	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8
0	-0,23	0,00	0,00	0,00	-0,96	-1,74	0,00	-0,21
2	-0,47	-0,32	-0,98	-0,93	-2,24	-2,34	0,03	-1,09
4	-0,34	-1,92	-1,96	-1,19	-1,93	-2,68	-2,79	-1,33
6	-1,36	-1,68	-2,88	-0,91	-1,99	-2,46	-2,41	-1,57
19	-0,44	-0,12	0,00	0,00	-0,24	-0,57	-0,52	-0,15
24	-0,92	0,00	0,00	-0,42	-0,02	-0,07	0,00	0,00

0,64%

Time [h]	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8
0	0,00	0,00	0,00	0,00	-0,70	-0,13	0,00	0,00
2	-0,21	-0,64	0,00	-1,08	-1,34	-1,15	0,00	-0,95
4	-0,42	-1,58	-0,30	-1,41	-1,10	-1,54	-2,34	-1,27
6	-1,26	-1,16	-0,73	-1,17	-0,95	-1,25	-2,24	-1,35
19	-0,22	-0,43	-0,16	-0,04	-0,73	-0,06	-0,76	-0,20
24	-0,54	0,00	0,00	-0,44	-0,57	0,00	-0,60	0,00

0,32%

Time [h]	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4	Volunteer 5	Volunteer 6	Volunteer 7	Volunteer 8
0	-0,85	0,00	0,00	0,00	0,00	-1,03	0,00	-0,45
2	-1,17	-1,51	0,00	-0,64	-0,41	-1,03	0,00	-1,13
4	-0,55	-2,41	-0,04	-0,44	-0,42	-1,15	-2,65	-0,79
6	-0,61	-1,60	-0,25	-0,47	-0,63	-0,92	-2,22	-1,40
19	-0,82	-1,25	-0,12	0,00	-0,27	-0,19	-1,07	-0,77
24	-0,37	-0,10	0,00	-0,15	0,00	0,00	-0,79	-0,40

Table 24: Baseline-adjusted, control site-corrected a-scale data following a 4-hour dose durations of BMV at different levels of the saturation level in MCT.

10%

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-0,90	-2,64	-1,72	-2,12	-0,48	-0,77
2	0,00	-0,68	-1,21	-1,21	-1,04	-0,45
3	-1,28	-1,11	-0,88	-1,22	-1,09	-0,34
4	-1,41	-1,22	-0,70	-1,56	-1,42	-0,36
5	0,00	-1,12	-0,91	-2,20	-0,40	-0,04
6	-1,20	-0,69	-1,63	-1,51	-0,55	-0,12

1,30%

Volunteer	0 h	2 h	4 h	6 h	19 h	24 h
1	-0,85	-1,46	-0,44	-1,81	-0,93	-0,14
2	-0,71	-0,28	-1,27	-0,65	-1,40	-0,07
3	-0,10	0,00	-0,81	-0,71	-1,07	-0,20
4	-0,03	-0,34	-0,54	-1,27	-0,48	-0,51
5	-0,39	-0,66	-0,47	-1,42	-0,70	-0,80
6	0,00	-0,75	-0,68	-1,57	-0,52	-0,67
7	0,00	-0,58	-0,93	-0,54	-0,44	-0,46

Table 25: Area under the response curve values (AARC) derived from the blanching response versus time profiles of BMV after different dose durations (DD). BMV concentration was adjusted to 80% of the saturation level.

	AARC DD 2h			
Volunteer	LMO	MCT	ME	TCL
1	3,37	-32,04	-57,32	-34,05
2	-10,01	-25,19	-31,93	-15,17
3	-4,77	-48,26	-42,63	-18,79
4	-16,55	-37,30	-47,83	-24,25
5	-16,84	-32,45	-49,47	-33,53
6	-8,51	-43,51	-36,61	-33,30

	AARC DD 3h			
Volunteer	LMO	MCT	ME	TCL
1	-10,47	-33,88	-36,37	-35,19
2	-20,33	-43,06	-48,52	-19,71
3	-41,20	-35,66	-29,36	-29,96
4	-11,88	-30,98	-49,65	-27,46
5	-36,70	-42,00	-56,05	-18,55
6	-11,71	-47,71	-31,32	-40,72

	AARC DD 4h			
Volunteer	LMO	MCT	ME	TCL
1	-17,01	-40,47	-53,00	-51,14
2	-24,62	-58,06	-46,55	-19,96
3	-26,54	-47,44	-43,62	-43,77
4	-18,15	-46,12	-29,98	-32,77
5	-20,86	-40,74	-50,76	-24,60
6	-39,97	-29,75	-49,58	-26,32

	AARC DD 5h			
Volunteer	LMO	MCT	ME	TCL
1	-5,38	-58,81	-47,75	-54,91
2	-23,76	-34,92	-49,40	-33,90
3	-26,69	-45,08	-51,20	-36,52
4	-28,99	-48,77	-48,67	-40,39
5	-26,42	-37,27	-47,04	-25,85
6	-41,73	-43,99	-45,17	-33,22

	AARC DD 6h			
Volunteer	LMO	MCT	ME	TCL
1	-37,75	-52,75	-45,67	-47,10
2	-21,69	-41,11	-48,29	-35,33
3	-36,82	-33,62	-57,78	-51,39
4	-29,58	-60,45	-70,48	-35,62
5	-25,61	-40,58	-40,98	-39,06
6	-28,28	-54,61	-51,63	-31,27

Table 26: Area under the response curve values (AARC) derived from the blanching response versus time profiles of BMV at different levels of saturation in ME after a 4-hour dose duration.

	AARC ME					
Volunteer	10,43%	5,21%	2,61%	1,30%	0,64%	0,32%
1	-52,17	-33,44	-15,46	-18,31	-14,04	-17,17
2	-49,62	-34,85	-22,30	-18,16	-17,01	-31,34
3	-49,98	-35,75	-26,92	-27,48	-7,51	-3,03
4	-43,53	-30,85	-37,90	-12,12	-15,22	-6,06
5	-48,89	-41,58	-54,95	-26,44	-20,70	-8,82
6	-40,74	-41,96	-33,31	-35,54	-15,43	-14,00
7	-48,54	-46,63	-27,23	-28,28	-29,82	-33,56
8	-32,76	-35,25	-17,12	-18,18	-16,37	-22,72

Table 27: Area under the response curve values (AARC) derived from the blanching response versus time profiles of BMV at different levels of saturation in MCT after a 4-hour dose duration.

	AARC MCT		
Volunteer	10%	1,30%	0,64%
1	-31,77	-29,45	-6,73
2	-23,34	-21,46	-12,14
3	-25,07	-17,18	-6,03
4	-30,63	-16,91	-6,50
5	-24,26	-24,10	-9,75
6	-22,42	-23,49	-6,75
7		-12,18	